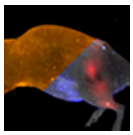
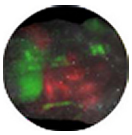
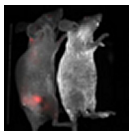
Maestro™ *In-Vivo* Fluorescence Imaging System



Detect multiple dyes, even when spatially colocalized.



Remove contrast-robbing autofluorescence.



Improve sensitivity and achieve results in seconds.

User's Manual for Maestro 2.2

August 2006

Notice

The information in this document is subject to change without notice and should not be construed as a commitment by Cambridge Research & Instrumentation, Inc. CRi assumes no responsibility for any errors that may appear in this document. This manual is believed to be complete and accurate at the time of publication. In no event shall CRi be liable for incidental or consequential damages in connection with or arising from the use of this manual.

This manual describes system operation using Maestro version 2.2 software.

For more information contact:

Cambridge Research & Instrumentation, Inc. (CRi)

35-B Cabot Road, Woburn, MA, 01801, USA

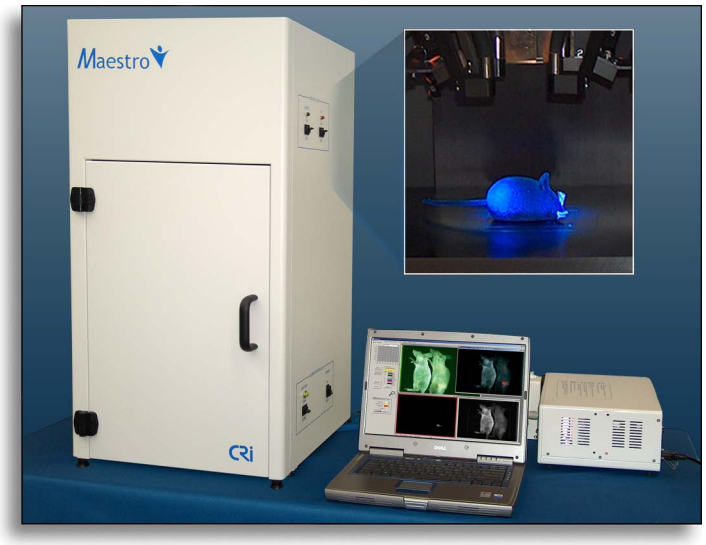
(Toll-Free US) 1-800-383-792 4, (Phone) +1-781-935-9099, (Fax) +1-781-935-3388

Email: techsupport@cri-inc.com or sales@cri-inc.com

Web site: http://www.cri-inc.com

Document Part No. MD15379 Rev. B, August 2006

Maestro™ *In-Vivo* Fluorescence Imaging System



CRi, 35-B Cabot Road, Woburn, MA, 01801, USA :: (Toll-Free US) 1-800-383-7924, (P) +1-781-935-9099, (F) +1-781-935-3388 :: sales@cri-inc.com :: www.cri-inc.com

Contents

Chapter 1, Introduction to Multispectral Imaging Theory and Concepts 1
Light....1
Human Perception of Light Intensity and of Color....2
A Highly Adaptive Light Detector....2
Response to Illumination....3
Ability to Distinguish Colors....3
Fluorescence....3
Stoke's Shift....4
Autofluorescence....5
Excitation and Emission Filters....5
The Multispectral Solution....6
Overlapping Emission Spectra....6
Multispectral Analysis....7
Solution for Imaging Small Animals....7

Chapter 2, Introduction to the Maestro *In-Vivo* Fluorescence Imaging System 9
Introduction....9
Operator and Equipment Safety....10
General Cautionary Statements....10
Illumination Module Cautionary Statements....10
For Technical Assistance....11
About This Manual....11
Design Change Disclaimer....12
Reproduction Disclaimer....12
CE, CSA, and UL Testing and Certification....12
Maestro Hardware Components....13
Imaging Module....13
System Power....14
Specimen Stage and Illumination Assembly....15
Fiber-Optic Illuminator Arms....16
Interior Lights....17
Emission Filter Slider....18
Illumination Module....19
Excitation Filter Slider....21
Switching the Illumination Module ON and OFF....21
Workstation Computer....22
Cleaning Your Maestro *In-Vivo* Imaging System....22
Launching the Maestro Application....22
Understanding the Maestro Work Area....23
Control Panels....23
Thumbnails and Image Data Pages....23
Window Layout....24
Resizing an Image Window....25
Zooming In and Out....25
Panning a Zoomed Image....25

Panning All Images Simultaneously 26
Specifying Camera Hardware Settings 26
Using Low Screen Resolution Mode 27
Reinitializing Maestro Hardware 28

Chapter 3, Imaging the CRi Business Card with QDot™ Spots **29**
First Steps 30
Acquiring an Image Cube 31
Unmixing the Image Cube 32
Calculating the Pure Spectra 34
Unmixing the Image 35
Quantifying Results 36

Chapter 4, Method Development **39**
Methods for Building Spectral Libraries 40
Tips for Spectral Library Development 40
Obtaining Autofluorescence and Label Spectra from a Single Animal 41
Obtaining Autofluorescence and Label Spectra from Two Animals 42
Saving Spectral Libraries 43
Saving Protocols 43
Importing Spectra Into a Library 44
From an Existing Library 44
From a Saved Component Image 44
From a Saved Composite Image 45
Practice Exercise 45

Chapter 5, Acquiring Spectral Images **49**
Tips for Obtaining Quantitative Results 50
Acquiring Images Using a Saved Maestro Protocol 50
Viewing a Live Image Stream 51
Selecting Camera Binning and a Region of Interest 51
Specifying the Current Wavelength and Exposure 52
Adjusting the Current Wavelength 52
Adjusting the Exposure Time 53
Specifying Custom Collection Settings 53
Selecting a Filter and Cube Wavelengths 54
Using Preset Filter Settings 55
Manually Entering Acquisition Settings 55
Modifying and Saving Preset Filter Settings 55
Acquisition Setup Using Multiple Filters 55
Maintaining Compatible Exposure Time Ratios 57
Saving Many Filter Protocols in One Maestro Protocol 57
Flat Fielding 58
Acquiring a Mono Image (Snap Shot) 58
Acquiring an Image Cube 58
Assigning a Sample ID to an Image Cube 59
Associating Notes with an Image Cube 59
Acquiring Timed Sequences of Image Cubes 59
Saving Images and Image Cubes 60

Saving Image Cubes Automatically....61
Viewing Cube Information....61
Extracting an Image Plane from a Cube....62

Chapter 6, Unmixing Spectral Images **63**
Unmixing with a Saved Spectral Library....64
Opening an Image Cube....64
Computing and Unmixing Spectra Automatically....65
Computing and Unmixing Spectra Manually....67
Sampling Spectra from a Cube....67
Selecting a Region of Interest Shape....68
Manually Computing Spectra....68
Error Scaling When Manually Computing Spectra....69
Using Fit Offset....69
Changing the Spectral Graph Scale....70
Saving An Unmixed Result Set....70
Working With Saved Result Sets....71
Importing a Cube Into a Result Set....71
Baseline Subtraction....72
Batch Data Processing....72
Using Line Profiles to Analyze Signals....74
Working with Line Profiles....76

Chapter 7, Quantifying Results **77**
Measuring Regions....77
Finding Regions Automatically Using Threshold Segmentation....77
Manually Drawing and Modifying Regions....79
Cloning Regions....80
Dragging a Copy of a Region to Another Image....80
Copying all Regions to the Clipboard....80
Saving and Loading Regions....81
Obtaining Accurate Measurements....81
Ignoring Smaller Regions....82
Hiding Region Labels....82
Adjusting Region Transparency and Color....82
Understanding Region Measurements....83
Copying Measurement Data to the Clipboard....84
Saving Measurement Data as a Text File....84
Appending Measurement Data....84

Chapter 8, Customizing Spectral Displays **85**
Adjusting Brightness and Contrast Levels....85
Returning to Display Defaults....86
Saturation Mask....86
Changing How a Cube is Displayed....87
Changing How a Composite is Displayed....88

Chapter 9, Frequently Asked Questions **89**

Appendix A, System Specifications & Dimensions **91**
Operating Specifications....91
Computer System Requirements....92
Mechanical Dimensions....93
Imaging Module....93
Illumination Module....94
Specimen Stage....95

Appendix B, System Setup & Installation **97**
Unpacking the Maestro *In-Vivo* Imaging System....97
Setting Up the Maestro *In-Vivo* Imaging System....99
Installing Maestro Software....101
Activating Maestro Software....103
Calibrating Pixel Size....104
Tests for Ensuring Proper Operation....105
Using Third-Party Computers with the Maestro Software....105

Appendix C, Filter Selection Guide **107**
Removing & Installing Filters in the Filter Slider....108
Installing Emission Filters into an Emission Filter Slider....108
Installing Excitation Filters into an Excitation Filter Slider....108
Removing Filters from a Slider....108
Cleaning Filters and Other Optical Surfaces....109

Appendix D, CRi Software End-User License Agreement **111**
Notice to Purchaser....113

Appendix E, Maestro Quick Start Guide **115**

Index **117**

Chapter 1

Introduction to Multispectral Imaging Theory and Concepts

This chapter provides an introduction to the theory and concepts that enable CRi's multispectral imaging systems to function as well as they do.

Topics in this chapter: **Page**

- Light 1
- Human Perception of Light Intensity and of Color 2
- Fluorescence 3
- The Multispectral Solution 6

Light

Light, as used in this discussion, means the portion of the electromagnetic spectrum that is visible to the human eye. While the physical description of light can be highly complex, we will restrict this discussion to the wavelengths of light, and the interaction of that light with physical and biological materials.

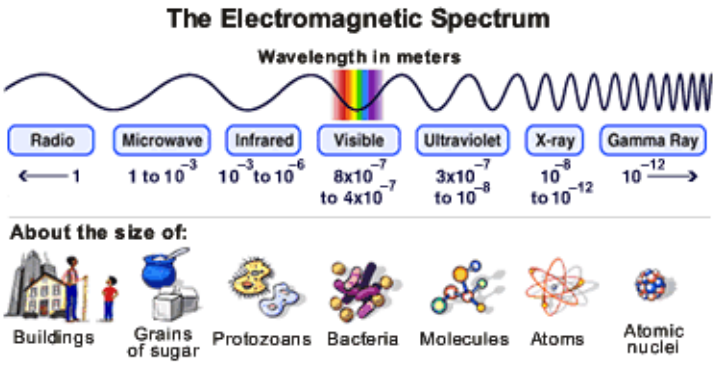


Figure 1. The Electromagnetic Spectrum[1]

The Electromagnetic Spectrum is illustrated here from radio to gamma ray frequencies. We are concerned with the visible wavelength range for purposes of this discussion, although some models of the Maestro *in-vivo* imaging system can operate out to 950 nm, into the so-called near-infrared wavelength range.

Although this illustration of the electromagnetic spectrum suggests that the visible range of light covers approximately 400 nm to 800 nm, most humans realistically are limited to the range of 400 nm (deep violet) to 700 nm (deep red). Visible light makes up a very small portion of the entire electromagnetic spectrum.

Light has energy. Visible light has an energy level of about one electron volt per photon. As one moves to shorter wavelengths of light, the energy per photon increases. In the shorter ultraviolet wavelengths, which approach soft x-rays, the electron energy per photon increases to 50 to 100 electron volts. This energy content of light is useful when one wishes to induce a change of energy state in a material (i.e., cause a receptive molecule to undergo a series of energy additions and then relaxations, resulting in fluorescence).

Human Perception of Light Intensity and of Color

A Highly Adaptive Light Detector

The human eye is a highly adaptive light detector, becoming significantly more sensitive in low light, and less sensitive under bright light. This adaptive change is not instantaneous, and it takes some time for the eye to fully adjust to a new illumination level. This is the reason one needs to "dark adapt" by being in a darkened room for some time before observing weak fluorescence through a microscope.

While the eye can be very sensitive to low light levels and can also "see" in very bright conditions, it does not discriminate light levels very well. An individual has no "internal

1. The Electromagnetic Spectrum illustration was prepared by NASA under Contract NAS5-26555 for the Education Group of the Space Telescope Science Institute's Office of Public Outreach. It is used here under public domain in accordance with NASA's contract.

meter" that indicates the current sensitivity level setting for the eye. The eye also has a limited ability to discriminate levels of illumination ranging from the lowest level to the highest level at any given sensitivity. US Department of Defense research indicates that while some people can distinguish as many as 500 levels of gray, most humans can only distinguish approximately 30 to 35 levels of gray, ranging from black to white. This is relatively insensitive to the actual total illumination level, although the ability to discriminate gray levels does degrade in both very dim light and very bright light.

Response to Illumination

The eye's response to illumination is not a linear response, but more closely approximates a logarithmic function. The result is that while the human eye interprets differences between gray levels as "even steps," to achieve a two-fold brightening of the perceived gray level, the actual illumination level would need to increase significantly more than a simple doubling.

Contrasting the eye with a digital electronic sensor system, any sensor that has 8-bit resolution can detect 256 levels of gray. As the number of bits of resolution increase, the number of gray levels also increases. A 10-bit system gives 1024 levels and 12 bits yields 4096 levels of gray. Digital electronic sensors are linear in response to light levels.

Ability to Distinguish Colors

While the eye is relatively poor at discriminating gray levels (intensity), it is very good at distinguishing colors. Most individuals are estimated to be able to discriminate thousands of colors. The problem is, no two individuals see precisely the same color. In other words, each individual interprets colors slightly differently when viewing the same color. The basis for this is the way in which color sensors are arranged in the eye.

The eye contains three different types of color sensors, similar in response to the red, green and blue detectors in color cameras. Individual colors are composed of some combination of responses from these three different types of color sensors. While the general arrangement of these color sensors (cone cells) in the eye is reasonably standard, there are differences in the total number of each type of cone cell, and in the actual physical arrangement within the detecting cell layer (retina). These minor variations lead to the differences in perceived color between individuals, as does the actual way in which the individual's brain learned to interpret color(s).

Fluorescence

Many biological and natural materials give off light of a particular color when exposed to light of another color. This property is called **luminescence**. If the emitted light occurs rapidly after illumination (around one-millionth of a second), the luminescence is called **fluorescence**. If the light emission takes longer than one-millionth of a second, the luminescence is called **phosphorescence**. Materials that exhibit fluorescence have proven extremely useful as labels or indicators in many biological systems.

Fluorescence light emission is different than light absorption. Each fluorescent molecule generates light. We measure the total amount of light generated, and are not dependent on the interaction of the light with another material, such as a dye. While it would seem that fluorescence is much more amenable to accurate measurement than absorbed light, there are a number of factors that complicate such measurements. Fluorescence is emitted by a molecule in all directions, and most imaging systems are designed to capture light coming from a particular direction only. Therefore, there is no way to capture all of the light emitted by a fluorescent molecule with such a system. Additionally, fluorescence emission is influenced by the local environment, in particular by pH. The total amount of fluorescence will therefore depend on these local conditions of pH, as well as other surrounding molecules that may either enhance or quench some of the fluorescence energy. There is also the problem of obtaining identical excitation of all fluorescence molecules in a specimen, and this can be exceedingly difficult to achieve.

Fluorescence is an extremely sensitive technique, as it is much easier to visually assess or measure light emission against a dark background than it is to see a decrease in light intensity from absorption by a dye. Regardless of the sensitivity of fluorescence, the difficult in establishing uniform excitation, and controlling for local environment effects makes quantitation of fluorescence emission difficult in biological preparations.

Stoke's Shift

Materials that fluoresce almost always emit light at a longer wavelength than the wavelength of the exciting light. As an example, rhodamine isothiocyanate can be excited by green light, and then emits red light. This difference between the wavelength of the exciting light and the emitted light is called the **Stoke's Shift** and is based on **Stoke's Law**.

A range of excitation wavelengths will excite fluorescence. This range of wavelengths is known as the **absorption spectrum**. The emitted light also covers a range of wavelengths, and this is known as the **emission spectrum**. Since the Stoke's Shift for most materials is not that great, there is generally some overlap between the excitation and the emission spectra. We will return to this point shortly as it does impact choice of emission and excitation filters in fluorescence systems.

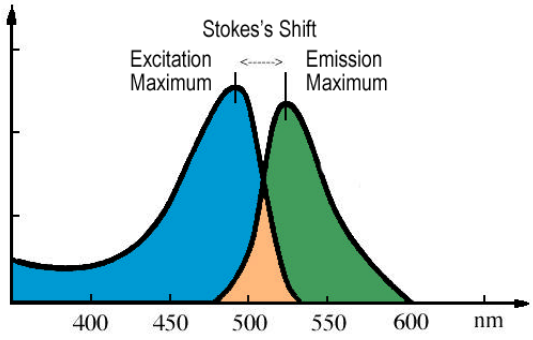


Figure 2. Stoke's Shift

Figure 2 contains an example excitation and emission spectra, showing Stoke's Shift and the overlap of the spectra.

Autofluorescence

Many biological materials are naturally fluorescent. In particular, many vitamins, some hormones, and a variety of biological enzymes and structural proteins are naturally fluorescent. These materials often fluoresce strongly enough to interfere with specific fluorescence labeling studies. Because of this unwanted background, or **Autofluorescence**, both excitation light sources and emitted light paths are highly filtered in fluorescence systems.

On continued stimulation (illumination at the excitation wavelength), most fluorescent materials fade. While some specific preparation methods can reduce the rate of fading, and different fluorescent materials fade at different rates, all fluorescent materials eventually fade, and this effect is irreversible. For this reason, specimens should be illuminated only while aligning and focusing, and during actual image collection. At other times, the excitation light should be closed off.

Excitation and Emission Filters

Filters that are used for fluorescence excitation and emission are specifically constructed to have very narrow bandpasses. They pass only a limited range of wavelengths of light. Restricting the excitation light wavelengths may reduce the amount of autofluorescence. Restricting the wavelength range of the emitted light helps minimize the amount of autofluorescence light that interferes with observing and measuring the desired specific fluorescence.

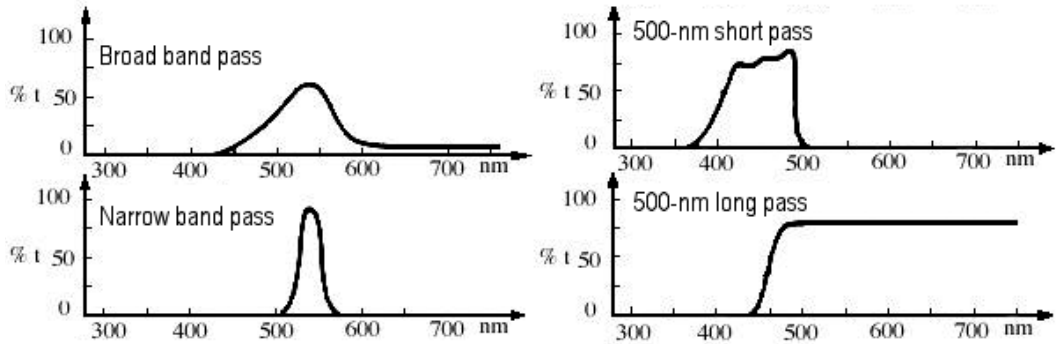


Figure 3. Excitation Filter Types

Excitation filters should be chosen to match the excitation maximum of the fluorescence label being used. The emission filter should match the emission maximum. In practical terms, the filter maxima may be slightly different from the ideal case, simply due to limitations of filter manufacturing, or to assist with autofluorescence reduction.

Specific excitation and emission filter combinations are available for most commonly used fluorescence dyes or labels. Nevertheless, regardless of how carefully one matches the excitation and emission filters to a given label, there will be some background autofluorescence, and this will reduce the perceived contrast between the “real” or actual label fluorescence and the specimen background.

The Multispectral Solution

The Maestro *in-vivo* imaging system offers a unique solution to the problem of autofluorescence and selection of emission filters. Multispectral analysis is based on the fact that all fluorescent materials produce a unique spectral emission. In other words, if you excite a material, and then examine the emitted fluorescence over a range of wavelengths, and record the intensity of emission at each point along the plotted curve of those wavelengths, you can generate an "emission spectrum" (like the green emission spectrum shown in Figure 2 illustrating Stoke's Law). This spectrum is different for each specific fluorescent material.

Overlapping Emission Spectra

The complication is that for many fluorescent labels of biological interest, the emission spectra overlap significantly, and these emission spectra may also be obscured by autofluorescence from other constituents of the specimen. Often, autofluorescence is a strong (bright) broad signal that may obscure the specific fluorescence that the investigator wishes to see.

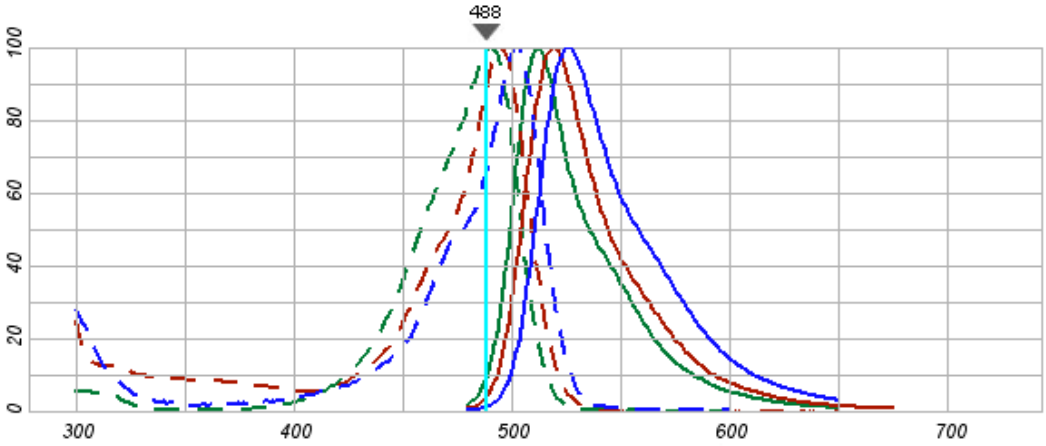


Figure 4. Overlapping Excitation and Emission Spectra

In this example of overlapping excitation and emission spectra, green is MitoTracker® Green Fn, Blue is Acridine Orange, and Red is Fluorescein isothiocyanate (FITC). The Cyan line at 488 represents the illumination light. Dotted lines are excitation spectra, and solid lines are emission spectra. (Illustration derived from Invitrogen™ Spectraviewer.)

There are three points to note in the graph shown in Figure 4.

- The excitation spectra overlap significantly with the emission spectra. This is why one needs to carefully select excitation filters. The goal is to prevent as much excitation light as possible from appearing in the emission spectra.
- Even with distinct emission spectra, there is significant overlap in the emission spectra of these three dyes. Visual examination of such a mixture of fluorescence spectra would be unable to distinguish these three dyes as individual "colors." They would be seen as some combination of yellow and green by most observers.

- The graphical display of spectra is normalized, and in actual practice, some fluorescent materials are much brighter than others.

As Figure 4 illustrates, many labels of biological interest have emission spectra that are so similar that separation using narrow band filters is difficult or impossible. Multispectral analysis provides the solution to this problem and reduces the need for multiple expensive narrow-band emission filters. A single long-pass emission filter can replace a large collection of emission filters.

Multispectral Analysis

The Maestro system's combination of unique hardware and sophisticated software make powerful multispectral analysis possible. Multispectral analysis generates spectral curves for the various fluorescent dyes or materials in a specimen. In addition, it generates a spectral curve for the autofluorescence that almost always is present to some degree.

In a multispectral analysis, a series of images are captured at specific wavelengths. The range of wavelengths captured should cover the spectral emission range of the labels present in the specimen. The number of images within that range should be chosen to adequately define the emission spectral curve for each label. The result will be a series of images, called an “image cube,” taken at specific wavelengths.

The data within the image cube is used to define the individual spectra of both autofluorescence and specific labels. Using sophisticated algorithms, the contribution of autofluorescence to the image can be removed, and the individual fluorescence spectra separated. The result is a set of images representing each spectrum that contributes to the final image.

In other words, as illustrated in Figure 4, multispectral analysis yields (1) an autofluorescence image, (2) a MitoTracker Image, (3) a Acridine Orange image, and (4) a FITC image. By removing the autofluorescence contribution to the image, the actual signals from the applied labels (MitoTracker, Acridine Orange and FITC) can be readily seen. If these individual images are recombined using highly contrasting colors to represent the location of each of the labels, a composite image of high contrast and readily observable colors can be generated.

Solution for Imaging Small Animals

Multispectral analysis also solves an even larger impediment to the imaging of small animals. Animal skin and fur is highly autofluorescent. In addition to the natural autofluorescence of skin, there is also distinct autofluorescence from the sebaceous glands (sebum) and from a variety of commensal organisms that may be present (mites, fungi, etc.).

Small animals may also demonstrate fluorescence from ingested food, particularly if the food contained chlorophyll (whose breakdown produces fluorescence strongly in the red). Multispectral analysis is able to separate all of these autofluorescence signals away from the specific labels applied to the specimen, and provides the ability to localize each material present, and to detect weak specific labeling even in the presence of strong autofluorescence.

Chapter 2

Introduction to the Maestro *In-Vivo* Fluorescence Imaging System

This chapter provides an introduction to the Maestro *in-vivo* imaging system. It includes a brief description of each of the system's hardware components and software features.

Topics in this chapter: **Page**

- Operator and Equipment Safety 10
- About This Manual 11
- CE, CSA, and UL Testing and Certification 12
- Maestro Hardware Components 13
- Launching the Maestro Application 22
- Reinitializing Maestro Hardware 28
- Understanding the Maestro Work Area 23
- Specifying Camera Hardware Settings 26
- Using Low Screen Resolution Mode 27

Introduction

CRi's Maestro *in-vivo* imaging system is a high performance multispectral imaging system designed for fluorescence macro-imaging. Maestro systems use an Imaging Module and an Illumination Module that enable fast and accurate imaging of specimens. The patented liquid crystal (LC) tuning element performs like a high-quality interference filter, which enables the transmitted light to be electronically tunable. Intuitive Maestro acquisition and analysis software runs on a powerful desktop or laptop computer.

There are two Maestro *in-vivo* imaging system models:

- The **Maestro VIS FL** operates in the 420 to 720 nm wavelength range.
- The **Maestro GNIR FL** operates in the 500 to 950 nm wavelength range.

Operator and Equipment Safety

It is the responsibility of the purchaser to ensure that all persons who will operate the Maestro *in-vivo* imaging system are aware of the following cautionary statements. As with any scientific instrument, there are important safety considerations, which are highlighted throughout this User's Manual.

General Cautionary Statements

READ AND UNDERSTAND THIS USER'S MANUAL BEFORE ATTEMPTING TO OPERATE, TROUBLESHOOT, OR MAINTAIN THE MAESTRO *IN-VIVO* IMAGING SYSTEM. READING THIS MANUAL FIRST MAKES IT EASIER AND SAFER TO OPERATE AND MAINTAIN THE SYSTEM.

Operate the system on a flat, stable surface.

Do not drop the imaging module or the illumination module.

Do not expose the imaging module or illumination module to prolonged heat above 40 °C.

Do not operate the system in an environment with explosive or flammable gases.

Do not subject the imaging module or its components to intense light from laser, focused arc or Hg lamp sources.

Do not operate the system in places where it may be splashed with liquid. (The imaging module may be cleaned with a sanitizing solution. Refer to "Cleaning Your Maestro In-Vivo Imaging System" on page 22 for cleaning guidelines.)

Use only the supplied power cables with three-prong plugs. Some cables and adapters supplied with the system have proprietary specifications. Do not connect components supplied by CRi using unqualified cables or adapters. Doing so could result in damage, and voids the Warranty.

Use only a properly grounded power outlet when connecting the system to power.

If you are using third-party mechanical components for the Maestro *in-vivo* imaging system, consult "Appendix A, System Specifications & Dimensions."

Follow the recommended maintenance procedures. This will help ensure optimal performance over years of use.

Caution! Servicing should be performed by CRi authorized and trained personnel only. Power must be disconnected from the system before servicing.

Illumination Module Cautionary Statements

The following cautions and warnings are printed on the illumination module.

Possible explosion hazard if used in the presence of flammable substances. Before disconnecting, read instructions. This warning pertains to the risk of explosion in the presence of flammable substances if the unit is unplugged from its electrical supply while the unit is on. Always switch the unit OFF before unplugging its power cord.

Caution: Disconnect power supply cord before servicing to avoid electric shock.

To reduce the risk of electric shock, do not remove cover. Refer servicing to qualified personnel.

Light source can cause permanent eye damage if viewed directly with unprotected eye.

End of light guide may be hot.

Keep cooling vents free from obstructions.

Endoscopic instruments used for this device must be certified to IEC 601-1 for medical equipment and IEC 601-2-18 for endoscopic equipment. This warning is not applicable to Maestro systems.

Caution: Do not remove lamp immediately after operation. Allow lamp to cool 10 minutes.

To prevent overheating, replace only with same type and rating of lamp. Read instructions before replacing lamp. See “Illumination Module” on page 19.

Do not operate light source without lamp in place.

For Technical Assistance

If you experience any difficulty setting up, operating, or maintaining your Maestro *in-vivo* imaging system, please contact your CRi representative. Office hours are 8:00 a.m. to 6:00 p.m. (Eastern Standard/Daylight Time), Monday through Friday.

- Telephone (US Toll-Free): 1-800-383-7924
- Telephone (Worldwide): +1-781-935-9099
- Facsimile (Worldwide): +1-781-935-3388
- Email: techsupport@cri-inc.com.

About This Manual

This manual is designed to serve users of both models of the CRi Maestro *in-vivo* imaging system using Maestro version 2.2 software. Operating instructions, functional descriptions, troubleshooting, illustrations, and other relevant information are contained in this manual.

Your Maestro system may include support documentation from third-party vendors. Bear in mind that components of the Maestro system may have been modified or custom-designed, so treat such third-party documentation as supplemental material only. In cases where CRI and third-party documentation differ, and you have any doubt as to which applies to your system, contact an authorized CRI distributor or service representative.

Design Change Disclaimer

Due to design changes and product improvements, information in this manual is subject to change without notice. CRi reserves the right to change product design at any time without notice to anyone, which may subsequently affect the content of this manual. CRi will make every reasonable effort to ensure that this User's Manual is up to date and corresponds with the shipped Maestro *in-vivo* imaging system.

Reproduction Disclaimer

No part of this manual may be reproduced, photocopied, or electronically transmitted, except for reference by a user of the Maestro *in-vivo* imaging system, without the advance written permission of CRi.

CE, CSA, and UL Testing and Certification

The Maestro *in-vivo* imaging system has been tested by an independent CE testing facility, and bears the appropriate CE mark.

The Maestro *in-vivo* imaging system has been awarded the right to display the CSA mark.

The Maestro *in-vivo* imaging system has undergone tests to meet UL standards.

The following are CRi distributors in the EU region authorized to function as primary contacts for CE-related matters concerning CRi products:

LOT-Oriel GmbH & Co. KG
Im Tiefen See 58
D-64293 Darmstadt
Germany
Tel: +49 6151 88 06 0
Fax: +49 6151 896667
Email: info@lot-oriel.de

LOT-Oriel UK
1 Mole Business Park
Leatherhead, Surrey, KT22 7BA
United Kingdom
Tel: +44 1372 378822
Fax: +44 1372 375353
Email: info@lotoriel.co.uk

Maestro Hardware Components

Imaging Module

The Maestro Imaging Module contains all of the principal imaging components in a single enclosure:

- High-resolution, scientific-grade CCD imaging sensor
- Solid-state liquid crystal (LC) wavelength tuning element
- Spectrally optimized lens and internal optics
- Emission filter assembly
- Flexible fiber-optic lighting system
- Light-tight enclosure with adjustable specimen stage and large door for easy access

The imaging module contains the following interface ports and indicator lights:

- USB Type A (rectangular) port
- USB 2.0 Type B (square) port for connection to the workstation computer
- Power connectors and fuse holders
- Switches for power and control of the flexible fiber-optic delivery system
- Anesthesia input and outlet ports

Figure 5 shows a front exterior view of the imaging module.

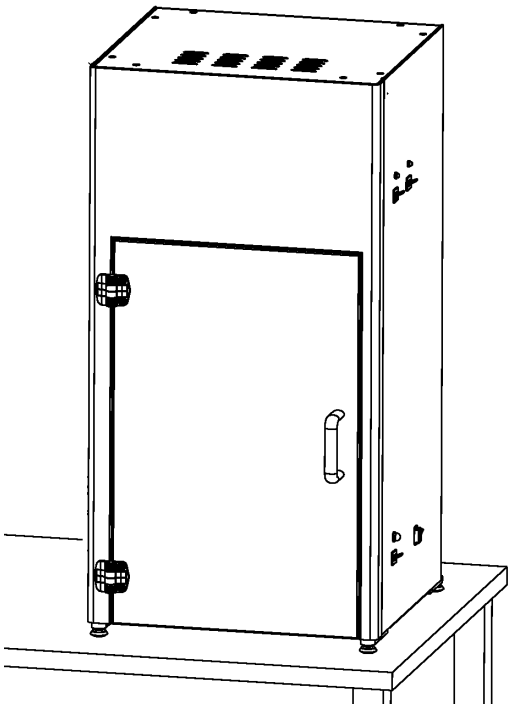


Figure 5. Maestro Imaging Module

System Power

On the rear of the imaging module there is a POWER ON/OFF switch that controls the imaging module's main power (Figure 6). Located immediately above the power switch is the fuse holder, which holds two 250VAC 8A fuses. These fuses prevent accidental circuit overloads in the imaging module.

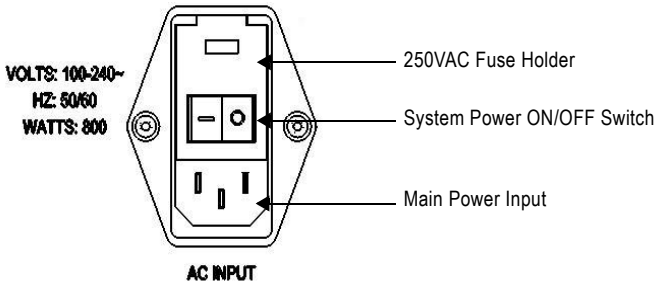


Figure 6. Main Power Switch

To switch power ON and OFF:

Switch the imaging module on by moving the POWER switch to the ON position. The imaging module will take approximately 45 seconds to initialize.

To replace fuses in the imaging module:

Use 250VAC 8A slow-blow Cooper Bussman® MDL-8 fuses.

1. Unplug the imaging module.
2. Locate the small black panel that covers the system fuse holder. It is located immediately above the main system power switch on the back of the imaging module.
3. Use a small screw driver to open the black fuse cover from the top and open it downward.
4. Use the small screw driver to gently pry the red fuse holder out of its port.
5. Replace both fuses in the fuse holder with 250VAC 8A slow-blow Cooper Bussman® MDL-8 fuses, available from CRi and other sources.
6. Replace the fuse holder into its port and close the cover.
7. Plug in the imaging module.

Specimen Stage and Illumination Assembly

The specimen stage and illumination assembly inside the imaging module can be set to nine discrete height positions. (There are three primary and six intermediate positions.) The selected height position determines the "field of view" that will be available when taking images. Lower the specimen stage for a larger field of view; raise it for a smaller field of view.

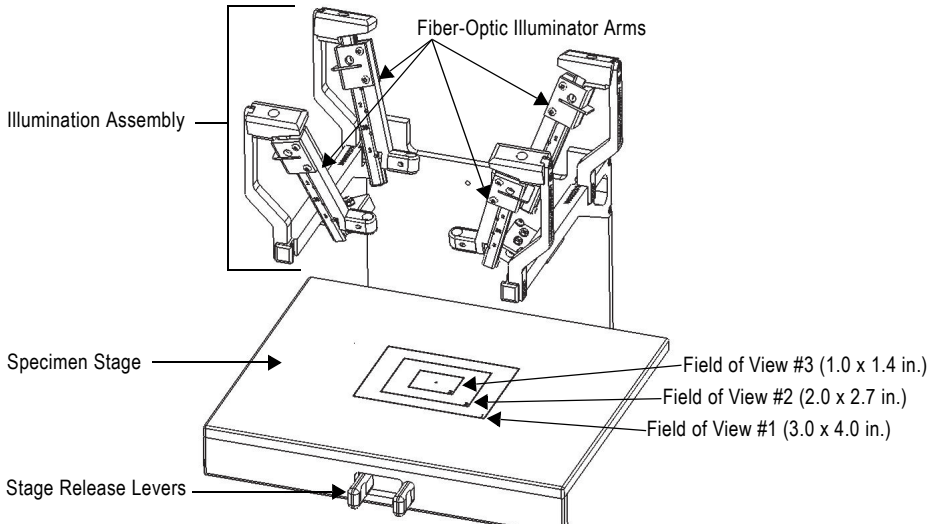

Fiber-Optic Illuminator Arms
Illumination Assembly
Specimen Stage
Field of View #3 (1.0 x 1.4 in.)
Field of View #2 (2.0 x 2.7 in.)
Field of View #1 (3.0 x 4.0 in.)
Stage Release Levers

Figure 7. Specimen Stage with "Field of View" Outlines

For reference, the specimen stage is etched with three numbered field of view outlines (shown in Figure 7). The height positions for the specimen stage are also numbered (shown in Figure 8).

The outlines correspond to the three primary stage heights: the lowest stage position (#1) gives the largest field of view (#1); the middle position (#2) gives the middle field of view (#2); the highest position (#3) gives the smallest field of view (#3). For example, to use the 3.0 by 4.0 inch field of view, set the specimen stage to position #1.

The intermediate stage positions between each primary position are labeled 1A, 1B, 1C, 2A, 2B, and so on. (To maintain clarity, these intermediates are not marked on the specimen stage.) Depending on your application, you may achieve better results using an intermediate stage height.

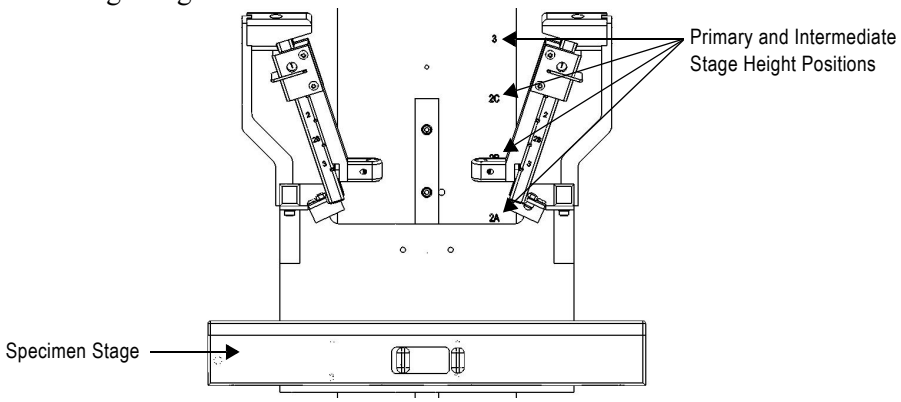


Figure 8. Stage Height Positions

To set the specimen stage height:

1. Squeeze the stage release levers on the front edge of the stage, then slide the stage up or down to the desired position.
2. Carefully release the tabs and ensure that the stage is locked at the desired position.
3. Move the fiber-optic illuminator arms (see below) to a position that corresponds to the stage height.
4. Set the corresponding stage height in the Maestro application. Select **Edit Hardware Settings** from the **Hardware** menu and select the **Camera Setting** tab in the **Edit Protocol** dialog box. Select the correct height in the **Stage Height** drop down box.

Fiber-Optic Illuminator Arms

Four fiber-optic illuminator arms provide adjustable and even illumination of the specimen. Move each of the illuminator arms to a height that corresponds with the height of the specimen stage. This will ensure consistent illumination of the specimen.

To position the illuminator arms:

1. Press the tab on the side of the arm to release it.
2. Slide the arm up or down to the position that matches the height of the specimen stage. Note that there is only one intermediate fiber-optic position for every set of three intermediate stage heights. You should choose the position that gives the best result for your application.
3. Set the corresponding lighting position in the Maestro application. Select **Edit Hardware Settings** from the **Hardware** menu and select the **Camera Settings** tab in the **Edit Protocol** dialog box. Select the correct position in the **Lighting position** drop down box.

Caution! The fiber-optic guides for the two illuminator arms closest to the operator are routed on the front (operator) side of the arms. The guides for the rear arms are routed on the far side of the arms. Be careful not to displace these guides during cleaning or other action. Doing so could limit the travel of the illuminators or the stage during the fiber-optic and stage setting procedure.

Interior Lights

On the right-hand side near the top of the imaging module, there are switches that control the interior lights. The switch labeled WHITE illuminates the visible wavelength lights and the switch labeled IR illuminates the near-infrared wavelength lights (see Figure 9).

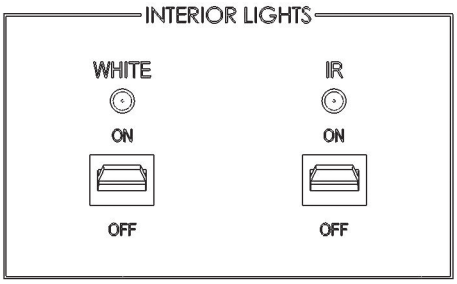


Figure 9. Interior Light (WHITE & IR) Switches

To switch interior lights ON and OFF:

Depending on your application, switch on either the WHITE or IR lights. The corresponding LEDs will illuminate (see Figure 10 for location of LEDs). Later, when you are ready to capture an image or image cube, remember to switch off the interior lights and switch on the excitation light.

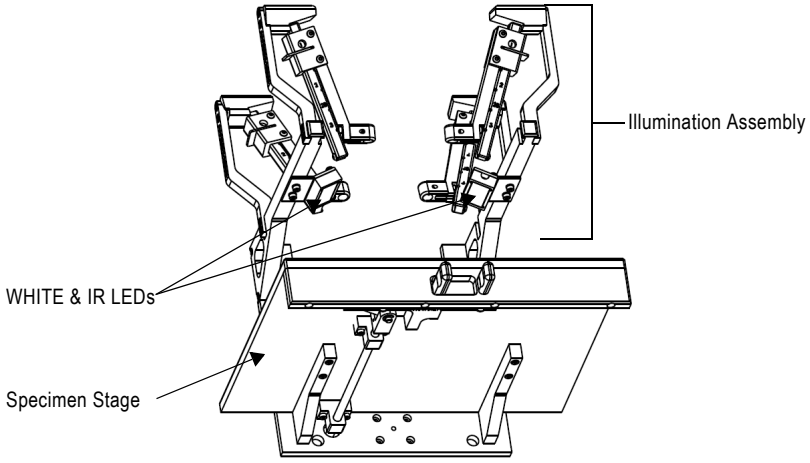


Figure 10. Position of Interior Light (WHITE & IR) LEDs

Note: When using the IR lights, you may not be able to see the near-infrared light emitted from the LEDs. This is normal because near-infrared light is not easily visible by eye.

To verify the IR interior lights are working:

When you switch on the IR interior lights, the IR indicator light above the switch should light up to indicate that the IR lights are on. If you want to visually verify that the IR lights are working, do the following:

1. Locate the IR LEDs by switching on both the IR and WHITE interior lights and looking for the white LEDs. The IR LEDs are located right next to the white ones (see Figure 10 for location).
2. Now switch off the WHITE lights. If you look very closely, you should see a faint red glow emitting from the IR LEDs. You may even need to dim other ambient lights to see this. If you see the faint red glow, the NIR illumination system is working properly.
3. If there is no glow, or if the IR indicator light is not lit, then contact CRi for repair.

Emission Filter Slider

The emission filter slider holder is located at the top interior of the imaging module and is shown in Figure 11.

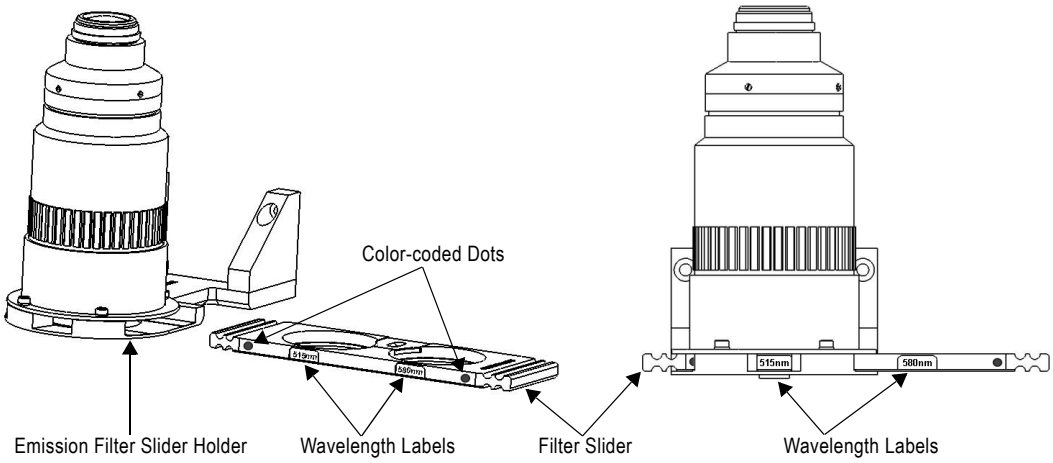


Figure 11. Emission Filter Slider Holder with Slider

The emission filter slider holds two 50 mm diameter longpass filters. Emission filter/slider sets ordered from CRi are color-coded to indicate their wavelengths. For example, the blue (515 nm) filter is indicated by a blue dot on its end of the slider, the green (580 nm) filter is indicated by a green dot, and so on. See “Appendix C, Filter Selection Guide” for detailed filter specifications.

To insert the slider:

1. Select the emission filter slider that contains the filter you want to use.
2. Insert the slider into the slot in the side of the emission filter slider holder (shown in Figure 11). The slider can only be inserted one way; the wavelength labels and color-coded dots must be upright and facing toward you.
3. Move the slider through the holder until the wavelength label for the filter you want to use is visible in the center window of the holder. You should feel the slider click into place when it reaches the proper position.

Illumination Module

The illumination module includes a Xenon light source, a fiber-optic delivery system, and power and interface cables.

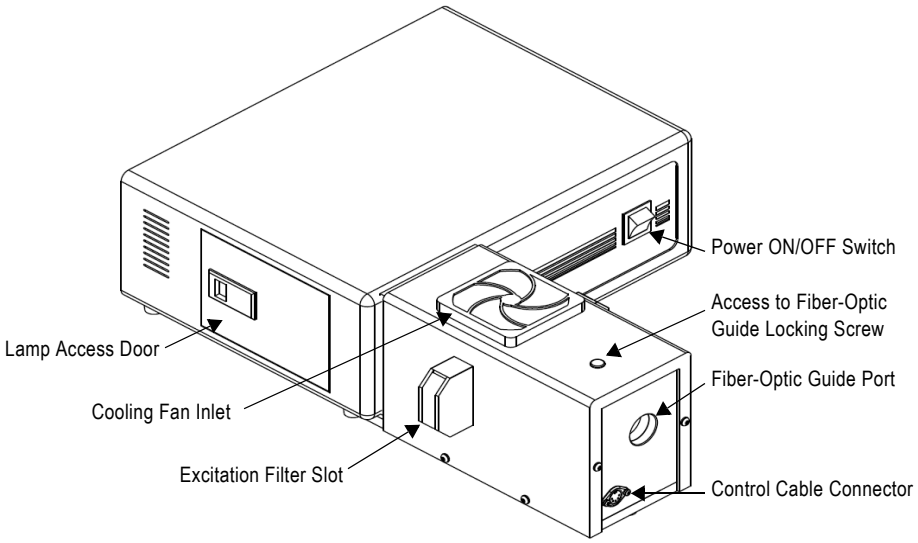


Figure 12. Maestro Illumination Module

The light source is a Cermax®-type 300 Watt Xenon lamp. It provides 5600 °K illumination with a typical lamp-life of approximately 500 to 1000 hours. After this time, the lamp intensity will be reduced by about 50%. If lamp brightness is important to your application, you should consider replacing the lamp during this time. An hour meter on the back panel of the illumination module indicates the number of hours the lamp has been illuminated. The housing of the lamp cartridge in your illumination module is colored either black or blue and can only be replaced with a cartridge of the same color. Contact CRi if you need to purchase a replacement, and indicate that you need a black (Part # OP10156) or blue (Part # OP10157) replacement lamp cartridge.

To replace the lamp cartridge:

Caution! Do not remove the lamp immediately after operation. Allow the lamp to cool for at least ten minutes. To prevent overheating, replace only with the same type and rating of lamp. Read all instructions before replacing the lamp. Do not switch the illumination module on without the lamp in place.

1. Switch off the illumination module and allow the lamp to cool for at least ten minutes.
2. Open the lamp access door (see Figure 12 for location) to expose the lamp cartridge.
3. Grasp the handles of the cartridge and pull firmly to remove the lamp.
4. Insert the new lamp, pushing it firmly into place. The lamp can only be inserted one way.
5. Close and latch the lamp access door.

6. Reset the hour counter by pressing the button directly beneath the counter. To press the button, first unscrew its cover. Then press the button and replace the cover.

To replace fuses in the illumination module:

Two 250VAC 8A slow-blow fuses in the illumination module prevent accidental circuit overloads. Fuses should be replaced with Cooper Bussman® MDL-8 fuses, available from CRi and other sources.

1. To replace a blown fuse, switch off power to the illumination module and unplug it.
2. Use a screw driver to turn the black fuse holder one half turn counter-clockwise and unseat it.
3. Remove and replace the old fuse in the holder. Reinsert the fuse holder into its port and turn one half turn clockwise until firmly seated.
4. Plug in the illumination module.

To clean the cooling fan inlet filter:

The filter for the cooling fan inlet should be cleaned on a regular basis to ensure the free flow of dust-free cooling air. Figure 13 shows the removal of the inlet filter.

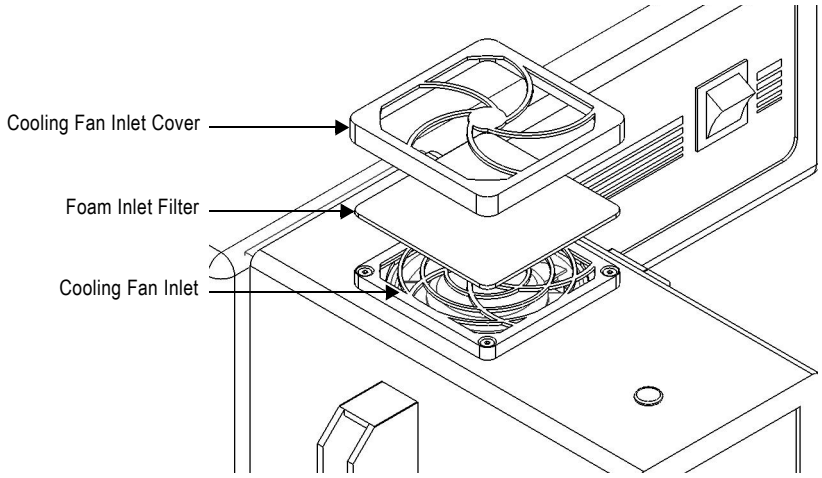


Figure 13. Removing the cooling fan inlet filter

1. Switch off power to the illumination module and unplug it.
2. Pry off the cooling fan inlet cover.
3. Remove the foam inlet filter and rinse it with mild soap and clean water.
4. Dry the filter thoroughly by gently squeezing out the water and pressing it with dry paper towels.
5. Reinsert the filter at the top of the cooling fan inlet and install the inlet cover.
6. Plug in the illumination module and switch on its power.

Excitation Filter Slider

The excitation filter slider holds two 50 mm diameter bandpass filters. Excitation filter/slider sets ordered from CRi are color-coded to indicate their wavelengths. For example, the blue (465 nm) filter is indicated by a blue dot on its end of the slider, the green (529 nm) filter is indicated by a green dot, and so on. See "Appendix C, Filter Selection Guide" for detailed filter specifications.

Note: The illumination shutter cannot be opened without a slider and excitation filter in place. This is to prevent the unfiltered light source from burning the end of the fiber-optic guide.

To insert the slider:

1. Select the excitation filter slider that contains the filter you want to use. Remember, the filters are color-coded, so if you installed the blue color-coded emission filter, use the blue color-coded excitation filter; if green, use green, etc.).
2. Insert the slider into the slot in the side of the illumination module (shown in Figure 12). There is no front-to-back orientation requirement for the excitation filter slider, but there is an up-down orientation. The filter keeper on the slider must be toward the top.
3. You should feel the slider click into place when the filter is properly located in the light path.
4. Once the slider with its filter is properly seated, the illumination shutter can be opened. If the illumination module SHUTTER and POWER switches are already in the OPEN and ON positions, the shutter will open automatically upon inserting the excitation filter.

Switching the Illumination Module ON and OFF

On the right-hand side near the bottom of the imaging module, there are switches and an indicator light for the illumination module shutter and power (see Figure 14).

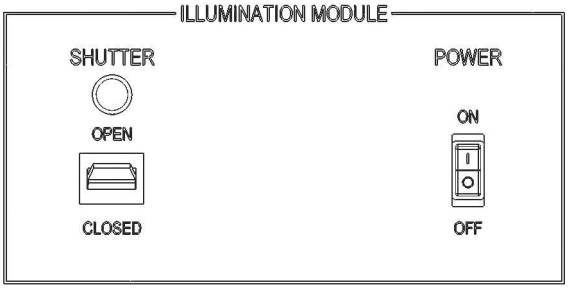


Figure 14. Illumination Module Power & Shutter Switches

After switching on the illumination module, it takes approximately fifteen to twenty minutes for the lamp output to stabilize to within 2% of its current maximum brightness.

Therefore, to achieve the most accurate and repeatable results, you should consider switching on the illumination module power when you first switch on the system. Then open the shutter when you are ready to acquire an image cube.

Remember to switch off the illumination module power when you will not use the system for a prolonged time period. After 500 to 1000 hours of illumination, the lamp intensity will be reduced by about 50%. If lamp brightness is important to your application, you should consider replacing the lamp during this time. Use the hour meter on the back of the illumination module to track the number of hours the lamp has been illuminated. Refer to "Illumination Module" on page 19 for lamp replacement instructions.

Workstation Computer

Maestro *in-vivo* imaging systems require a computer with a level of performance appropriate for acquiring and analyzing digital multispectral data. Computers purchased from CRi that will be used with the Maestro system meet the performance requirements listed in "Computer System Requirements" on page 92. If you decide to supply your own computer, be sure it meets the performance requirements as well.

Cleaning Your Maestro *In-Vivo* Imaging System

It may be necessary to periodically clean the inside and outside of your Maestro *in-vivo* imaging system. Follow these guidelines when cleaning:

- Spray a clean cloth with a sanitizing solution such as 70% ethanol and wipe down the specimen table and specimen pad. Wipe all surfaces below, around, and behind the specimen stage as well.
- Be careful never to spray directly into the imaging module to prevent spraying the lens, the camera, or the fiber-optic tips.
- Wipe down all external surfaces with the same solution and a clean cloth.

Launching the Maestro Application

If you have already purchased the Maestro system, your Maestro software was installed and activated for you at the factory. Double-click the **Maestro** icon on the workstation computer's desktop to launch the Maestro application.

If you need to install and/or activate the Maestro software, see "Installing Maestro Software" on page 101.

To evaluate Maestro software:

If you have not yet purchased a Maestro system but you want to evaluate the Maestro software, select the "I want to evaluate Maestro..." option when the Activation dialog box opens. You will be allowed to use the Maestro software for 30 days before being required to purchase and activate the software to continue using it.

Understanding the Maestro Work Area

The Maestro application's work area includes a menu bar, a toolbar, control panels for acquiring and analyzing images, an image view area, and thumbnails and data pages. Figure 15 shows a sample of the work area with an open image cube window.

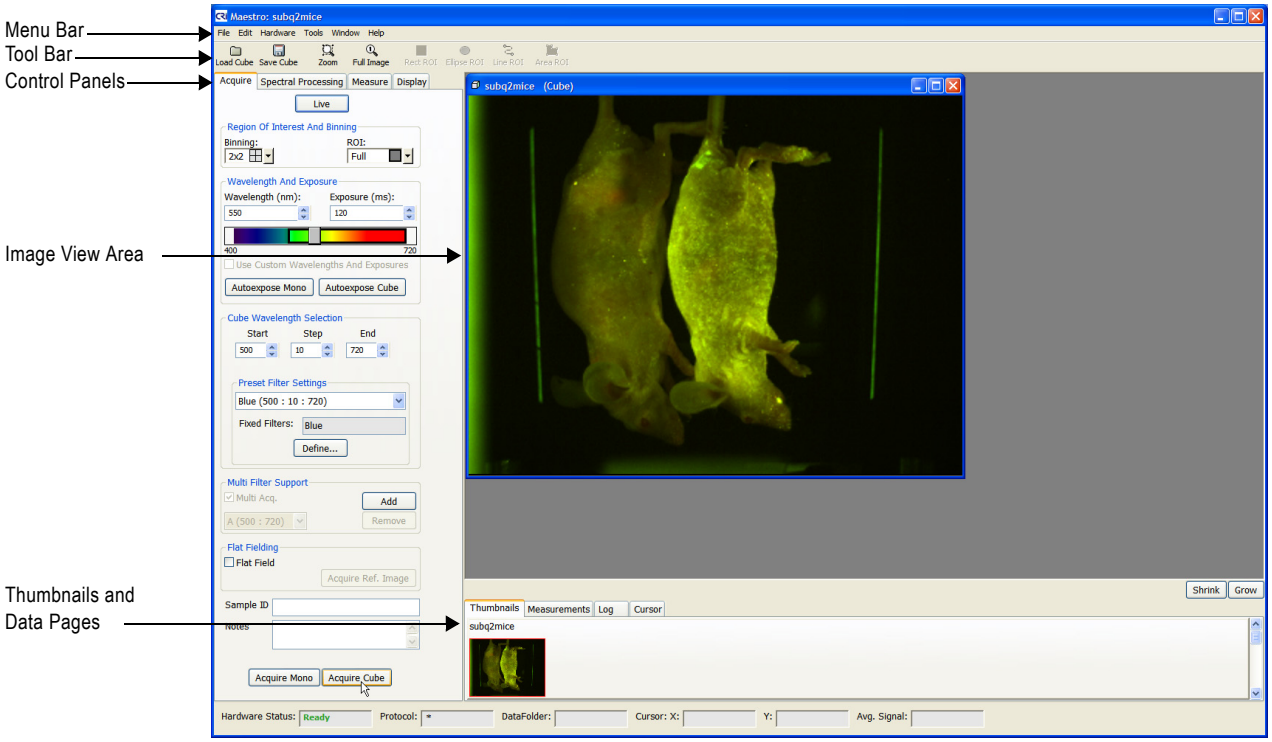


Figure 15. Maestro Work Area

Control Panels

To select a control panel:

Click the control panel's tab. The following panels are available:

- The **Acquire** panel includes all the controls needed to acquire new spectral images and image cubes.
- The **Spectral Processing** panel is used to create spectral libraries and unmix cubes.
- The **Measure** panel is used to find, measure, and adjust measurement regions in component images.
- The **Display** panel is used to change current display settings of images.

Thumbnails and Image Data Pages

There are four pages with tabs across the bottom of the image viewing area. You can change the viewable size of these pages by clicking the **Shrink** or **Grow** buttons at the far right (see Figure 15). These pages display the following types of information:

- **Thumbnails.** This page displays a thumbnail of each open image in the image viewing area. You can click on any thumbnail to activate its image window. If you have zoomed in on an image, the zoom view rectangle within the thumbnail shows the zoomed region. (You can drag this rectangle with the mouse pointer to pan the zoomed image.)

Figure 16. Thumbnails Page

- **Measurements.** This page displays the measurement data of measurement regions drawn on component images. See also, “Chapter 7, Quantifying Results.”

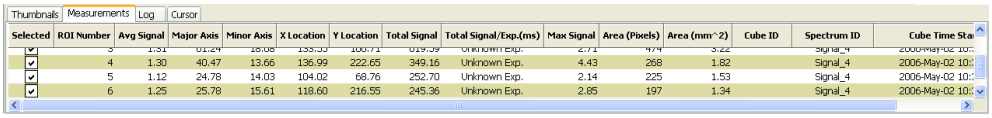


Figure 17. Measurements Page

- **Log.** This page contains an action history of the current Maestro session. The log can be saved or cleared by clicking the buttons at the far right of the page.

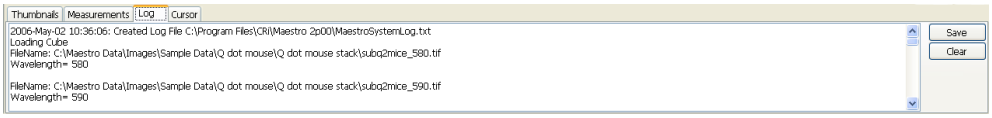


Figure 18. Log Page

- **Cursor.** This page provides information about the pixel directly below the cursor. For cubes, it displays the average signal. For composites, it displays absolute and percent contribution from each component. For other image types, it displays the pixel values.

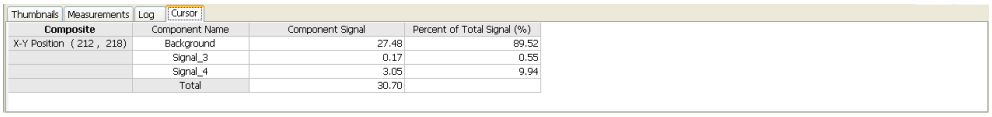


Figure 19. Cursor Page

Window Layout

Each window type has a home position on the screen. The cube lives in the upper left corner, the composite image lives in the upper right corner, and the component images are positioned in a grid starting from the lower left and working up and to the right.

Pressing Ctrl+L will force the windows to their home positions. In addition, many operations will force the windows to their home positions. These include resizing the thumbnail view, unmixing a cube, or activating a window from the thumbnail view.

Resizing an Image Window

There are a few ways to change the size of an image window or windows.

To manually resize a window:

Click and drag its window border to the desired size. The image inside the window resizes to fill the window, while also maintaining its aspect ratio.

To maximize the current window:

Click the window's maximize button. The window will fill the image viewing area.

To expand or reduce a window in steps

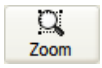
Select **Expand Window** (or press Ctrl+E) or **Reduce Window** (or press Ctrl+R) from the **Window** menu.

Zooming In and Out

You can zoom in to focus on details or zoom out to see more.

To zoom in:

Click the **Zoom** button on the toolbar. The mouse pointer changes to a magnifying glass. Use the magnifying glass to draw a box around the area you want to zoom.



You can also click the area of the image you want to zoom. Each time you click the image with the Zoom tool, the zoom increases. In addition, you can scroll the mouse wheel to zoom in.

To zoom out:

Click the **Full Image** button on the toolbar.

You can also right-click on a zoomed image and select **Zoom To Full Image** from the pop-up menu. You can also scroll the mouse wheel to zoom out.

Panning a Zoomed Image

When you zoom in on a particular area of an image, you might not be able to see other areas of the image.

To pan a zoomed image:

Do one of the following:

- Right-click on the image and select **Pan** from the pop-up menu. The mouse pointer changes to a hand icon. Click and hold the mouse button while dragging, or panning, to view the desired area of the image.

- Drag the zoom view rectangle that appears on the corresponding thumbnail image in the **Thumbnails** tab.
- Scroll the mouse wheel while holding down the Shift key (to pan horizontally) or the Control key (to pan vertically).

Panning All Images Simultaneously

Zoom in on an image, then hold down the Shift or Control key and drag the zoom view rectangle on a thumbnail image. All images pan simultaneously.

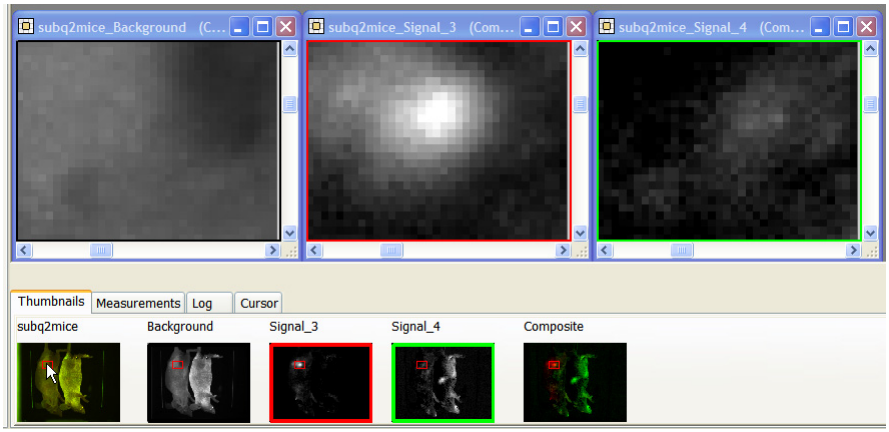


Figure 20. Simultaneous Panning

Specifying Camera Hardware Settings

The **Camera Setting** tab of the **Edit Protocol** dialog box lets you specify a variety of settings and parameters for the camera in your Maestro *in-vivo* imaging system.

To access these controls, select **Edit Hardware Settings** from the **Hardware** menu and select the **Camera Setting** tab. A brief explanation of each control is described below:

- **Filter Serial Port** can be used to select an alternate connection port for the Maestro hardware. The autodetect utility should select the correct port automatically, but this feature lets you override the default setting if necessary.
- **CCD Bit Depth** lets you capture either 8-bit (256-level) or 12-bit (4096-level) grayscale images per wavelength. 12-bit images are twice as large as 8-bit images and can only be opened using scientific imaging applications, including Maestro. In most instances, it is best to use the default 12-bit selection, as this gives higher quality images. Many Microsoft® Windows® applications cannot open 12-bit images. Therefore, if you want to capture images that can be displayed using non-scientific imaging applications, then select the 8-bit option.
- **Gain** affects image quality because it modifies the CCD readout process. For images that have low intensities, consider increasing the gain value slightly to achieve images with brighter pixel values. Increasing the gain can also reduce the exposure time. Note, however, that higher gain settings can result in increased signal noise,

which reduces image quality. Gain values of 1, 2, or 3 should be suitable for most applications.

- **Check Dark Current** is used to calculate the amount of background noise present in the camera. This is calculated using a zero time exposure and no light reaching the camera.
- **Frames to Average** is a useful feature for reducing noise in acquired images by increasing the signal-to-noise ratio. This value specifies the number of images the camera will take at each acquisition wavelength. The software then averages the values of those images to create a single image for each wavelength.

  The signal-to-noise ratio increases approximately by the square root of the number of images averaged. For example, averaging four images per wavelength gives a 2x signal-to-noise ratio. This should translate into approximately a 2x reduction in the limit of detection.
- **Autoexpose Target % of Dynamic Range** sets the autoexposure target values, and attempts to prevent saturation of pixels by restricting the exposure time to a percentage of the dynamic range of pixel values. For example, the default value of 70% for 12-bit (4096-level) images results in a maximum intensity value of 2867.
- **Maximum Exposure** lets you specify a maximum exposure time. The default value is 5 seconds.

Using Low Screen Resolution Mode

Maestro software only supports screen resolutions of 1280x1024 or better. However, many projectors do not support resolutions this high. The Maestro software has a special mode to support projectors or screens at 1024x768 resolution. Some of the Maestro software's functions will not be available in this mode.

Screen resolution is detected at time of startup. A dialog box warns you if you are running in low-resolution mode. If the Maestro software is started, and subsequently the screen resolution is changed, the Maestro software will not detect it.

If the Maestro software is run with a screen height of 800 pixels or less, it makes the following adjustments:

- In low-resolution mode, spectral libraries can only have five elements. This will effect the **Spectral Processing** and **Display** panels as well as the **Batch Mode**, **Import Spectral Library**, and **RCA** dialog boxes.
- The toolbar is removed from beneath the main menu.
- The **Define** button for **Filter Selection** is removed from the **Acquire** panel.
- The **Sample ID** and **Notes** boxes are removed from the **Acquire** panel.
- The **Notes** box is removed from the **Spectral Processing** panel.
- Plots on the **Manual Compute Spectra** dialog box are 4/5 their normal size.
- The spectral graph is removed from the **Display** panel.

Reinitializing Maestro Hardware

When you launch the Maestro application, it automatically detects the Maestro hardware (imaging module) if it is attached to the computer via the USB 2.0 cable. If the Maestro application loses its connection to the Maestro hardware, make sure the USB 2.0 cable is properly connected to the imaging module, then select **Reinitialize** from the **Hardware** menu. The Maestro application should detect and initialize the hardware. If this fails to reinitialize the connection, try restarting the Maestro software.

Chapter 3

Imaging the CRi Business Card with QDot™ Spots

This chapter steps you through the process of acquiring an image cube of the CRi business card with three QDot (quantum dot) spots painted on its front surface. (This business card was included with your Maestro *in-vivo* imaging system.)

We will also practice saving, unmixing, and analyzing the component and composite images. By following the instructions in this chapter, you will quickly learn how to use the Maestro *in-vivo* imaging system, as well as verify that the system is working properly.

Topics in this chapter: **Page**

- First Steps 30
- Acquiring an Image Cube 31
- Unmixing the Image Cube 32
- Quantifying Results 36

First Steps

1. Before you begin, verify each of the following connections are complete (refer to "Appendix B, System Setup & Installation" if necessary):
   - The imaging module is plugged into a surge-protected electrical outlet.
   - The USB interface cable is connected between the workstation computer and the imaging module.
   - The illumination module is connected to the imaging module with its interface cable, and its power cord is plugged into the accessory outlet on the back of the imaging module.
2. Switch on the imaging module, the illumination module, and the workstation computer, and switch on the WHITE interior lights.
3. On the workstation computer, launch the Maestro application.
4. The CRi business card test specimen has three QDot spots applied to its front surface: 565 nm towards the left and 605 nm towards the right, and a mix of both 565 and 605 nm dots in the center. The excitation wavelength is 465 nm. Therefore, we will use the blue Maestro filter set for this exercise.
5. Insert the blue excitation filter in the illumination module. The section, "Excitation Filter Slider" on page 21 explains how to install the excitation filter slider.
6. Open the door of the imaging module and install the corresponding blue emission filter in the imaging module. The section, "Emission Filter Slider" on page 18 explains how to install the emission filter slider.
7. Set the f/stop position for the camera lens at 2.8. The f/stop selector is located near the top of the lens assembly, just below the ceiling inside the imaging module.

Note: For most applications, the f/stop should be set at 2.8. If there is a need for greater depth of focus, then set the f/stop to the smallest value that will give an acceptable depth of focus. Remember to return the f/stop to 2.8 when you are finished with an alternate setting.

8. Move the specimen stage and illuminator arms to position 1B. ("Specimen Stage and Illumination Assembly" on page 15 describes how to move the stage and illuminator arms.)
9. In the Maestro application, select **Edit Hardware Settings** from the **Hardware** menu and enter the new stage height and lighting settings on the **Camera Setting** tab.
10. Place the CRi business card on the center of the specimen stage, within the marked viewing area.

Acquiring an Image Cube

Select the **Acquire** panel (if not selected already) by clicking its tab.

Before you begin, note the **Hardware Status** bar at the bottom of the work area. Verify that "Ready" is displayed in the status box. If the status box displays "No Hardware," verify that the interface and power cables are properly connected and that the imaging module is turned on.

1. If you don't already see the Live Stream window in the image viewing area, click the **Live** button at the top of the **Acquire** panel. A live image of the business card opens in the Live Stream window.

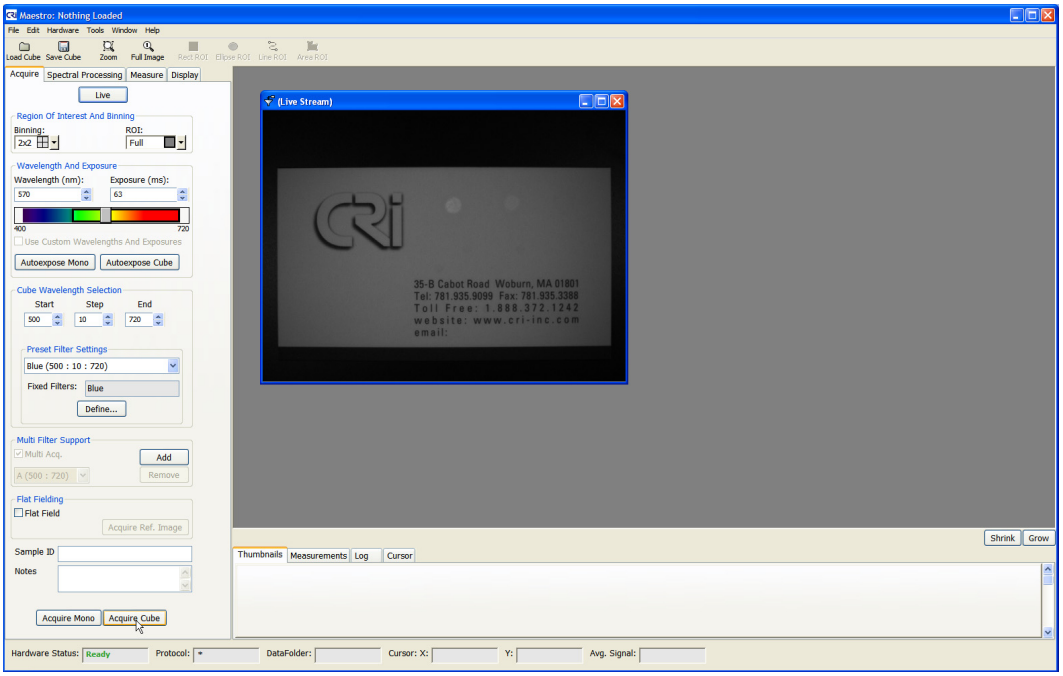


Figure 21. Acquire Panel with Live Grayscale Image

2. In the **Wavelength and Exposure** box, set the **Wavelength** to 570 nm and click the **Autoexpose Mono** button to improve the live image. The calculated exposure setting will display in the **Exposure (ms)** text box.

3. To adjust the position of the business card on the stage and focus the image, open the imaging module door and position the card so it is clearly visible in the Live Stream window. Then focus the lens to achieve a sharp live image.

Note: When you open the door of the imaging module, you may notice that much of the business card image turns red. This is caused by excess light entering the imaging module and causing the image to saturate. This will correct itself when you close the imaging module door. If the image is so saturated that you are unable to focus, try autoexposing the image while the door is open. This will reduce the exposure time and the saturation.

4. Close the imaging module door. Click the **Autoexpose Mono** button again if you had to change the exposure setting when focusing.
5. Accept the default **Binning** (2x2) and **Region Of Interest** (Full) settings for now.
6. In the **Preset Filter Settings** box, select the "Blue (500 : 10 : 720)" filter set from the drop down box. This automatically populates the **Start**, **Step**, and **End** wavelength settings.
7. Switch off all interior lights. The specimen stage should now be illuminated with blue light. (You can open the door of the imaging module to verify this.)
8. In the **Wavelength and Exposure** box, click the **Autoexpose Cube** button to automatically calculate the exposure settings for the image cube.
9. Now, to acquire an image cube, click the **Acquire Cube** button at the bottom of the **Acquire** panel.

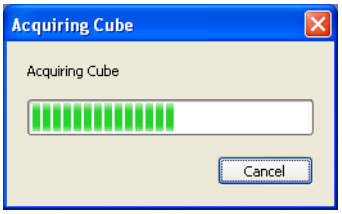


Figure 22. Acquiring Cube

10. When the Maestro system has finished taking the image cube, a color representation of the cube will be displayed in the image viewing area.
11. Click the **Save Cube** button on the toolbar to save the new image cube. In the **Save Cube** dialog box, select a location and name for the image cube and click the **Save** button. For this exercise, name the cube: *"qdotcard."*
12. You are now ready to unmix the fluorescence signals from the autofluorescence in the image cube.

Unmixing the Image Cube

1. Enlarge the cube view window by clicking once on the window to make sure it is active and then press Ctrl+E two times.
2. Switch to the **Spectral Processing** panel.
3. Sample the autofluorescence signal:
    a. Click the **Draw** button in the first row of the color palette and use the mouse pointer to sample (by clicking and drawing a line through) an area of autofluorescence on the business card (see Figure 23). A check mark appears in the **Select** box for row #1.

b. Choose the Black color marker from the **Color** drop down box in row #1. (Notice the sampling line turns black.) Unmixing the autofluorescence into Black will cause it to "disappear" in the unmixed composite image.

c. In the **Name** column of the color palette, rename this spectrum from *"C1"* to *"Paper."*

4. Next, use the Green color marker to sample the QDot spot that contains the 565 nm fluorescence plus the paper. This is the dot on the far-left side.

a. Click the **Draw** button in the second row of the color palette and use the mouse pointer to sample an area (by drawing a line) within the green QDot spot (see Figure 23).

b. In the **Name** column of the color palette, rename this mixed spectrum from *"C2"* to *"Q565+Paper."*

5. In the third row of the color palette, use the Red color marker to sample the QDot spot that contains the 605 nm fluorescence plus the paper. This is the dot on the far-right side. Name this mixed spectrum *"Q605+Paper."* Your work area should look similar to Figure 23.

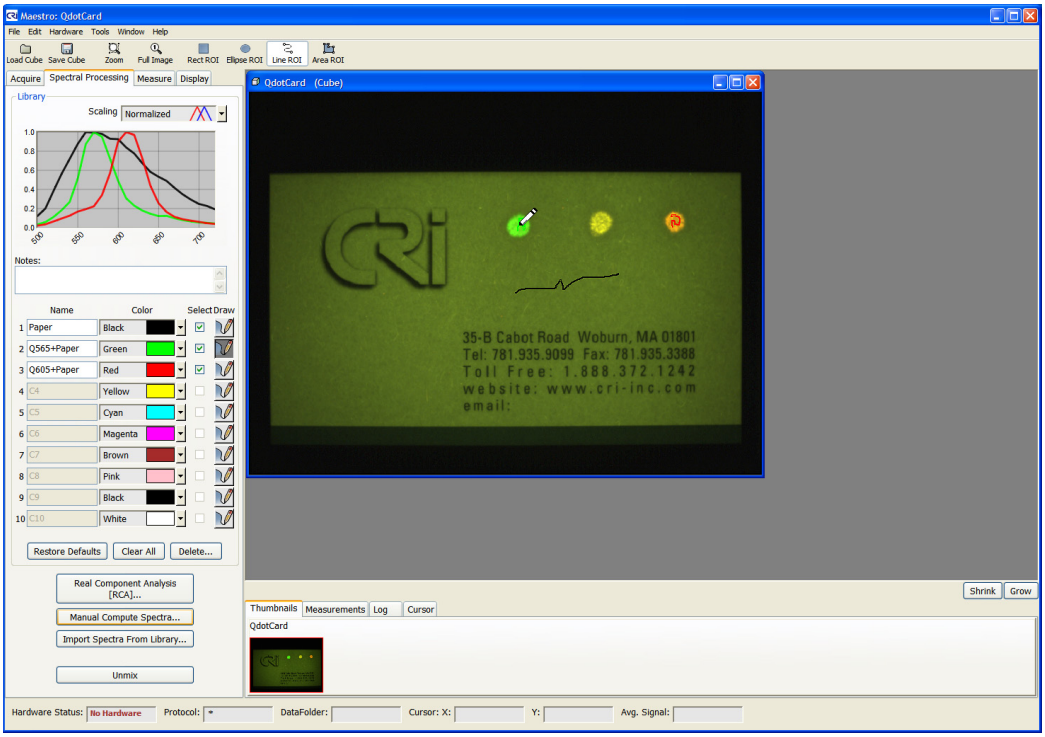


Figure 23. Image Cube with Sampled Spectra

Note: Notice the overlapping spectra in the spectral graph at the top of the **Spectral Processing** panel.

Calculating the Pure Spectra

1. Click the **Manual Compute** button located below the color palette to open the **Manual Compute Spectra** dialog box.
2. Separate the 565 nm QDot signal from the autofluorescence:
   a. In the **Known Spectrum** box, click the black **Paper** color marker.
   b. In the **Mixed Spectrum** box, click the green **Q565+Paper** color marker.
   c. In the **Computed Spectrum** box, make sure "Scale To Max" is selected in the **Scaling** drop down box.
   d. You will see the mixed spectrum in green and the computed spectrum in yellow (the color selected in the color drop down box at the bottom of this dialog box). The dotted "best fit" line is a scaled representation of the known (autofluorescence) signal.
   e. To obtain an accurate pure spectrum (which is required for an accurate unmixing), one must subtract the correct amount of autofluorescence from the mixed spectrum. To do this, it is important that the non-overlapping regions between the "pure" fluorophore and the autofluorescence line up closely. To do this:
      - First, try sliding the **Error Scaler** to the right (or left) until the spectra are aligned as closely as possible. (The **Scale** option is discussed further in "Error Scaling When Manually Computing Spectra" on page 69.)
      - If the non-overlapping regions do not line up closely using scaling, try clearing the **Scale** check box, and observe the results, which should be similar to the plot shown in Figure 24. For this exercise, scaling should not be necessary because of the strong QDot signal.
      - Note that the computed spectrum looks more like a simple gaussian curve, which is the shape of accurate quantum dot spectra. The "wings" on either side in the original mixed spectrum, which are due to the contribution of the paper autofluorescence, have been removed.

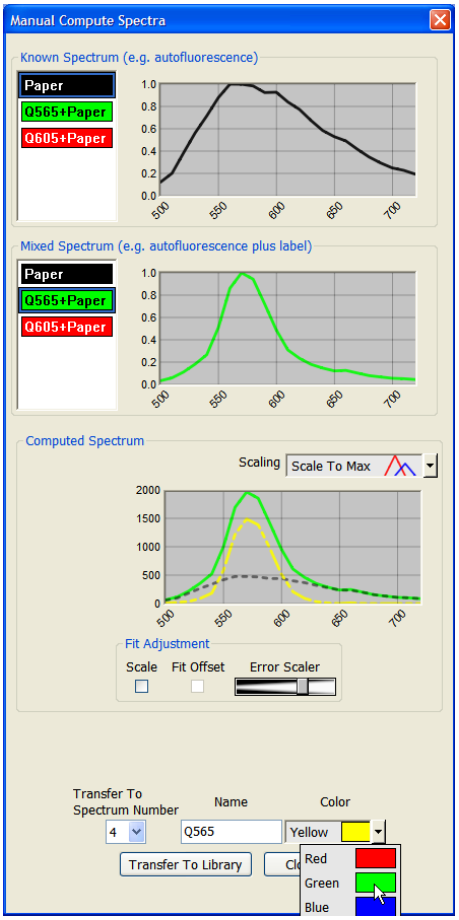


Figure 24. Manual Compute Spectra

f. At the bottom of the dialog box, change the name of the computed spectrum from *"C4"* to *"Q565."* For simplicity, change its color to green to correspond with the green QDots.

g. Click the **Transfer to Library** button. You will see the new selection added to row #4 of the color palette.

3. Now separate the 605 nm QDot signal from the autofluorescence:

   a. In the **Known Spectrum** box, the black **Paper** color marker should still be selected.

   b. In the **Mixed Spectrum** box, click the red **Q605+Paper** color marker.

   c. If necessary, adjust the **Error Scaler** or turn scaling off to accurately compute this spectrum.

   d. At the bottom, change the name from *"C5"* to *"Q605."* Change its color to red to correspond with the red spot of QDots.)

   e. Click the **Transfer to Library** button.

4. Close the **Manual Compute Spectra** dialog box. Notice that the check boxes in the **Select** column for each of the mixed signals were automatically cleared when you added the pure spectra selections to the color palette.

5. Now you are ready to unmix the image and compute the pure spectra.

Unmixing the Image

1. Make sure only the **Paper**, **Q565**, **Q605** color markers are selected in the color palette.

2. Click the **Unmix** button at the bottom of the **Spectral Processing** panel.

3. The Maestro software calculates and displays images for the pure fluorescence (QDot) signals and the autofluorescence (paper) signal. A new set of images appears in the image viewing area.

   Figure 25 shows the grayscale images of each signal, as well as a new composite color image created using the colors you selected.

   - The original image (cube) is shown at the top left.
   - An RGB color-composite image of the pure signals is shown in the top right. (We cannot see the autofluorescence because it was unmixed into black.)
   - Images representing each of the pure signals are shown across the bottom in grayscale. Their window borders are color-coded to correspond to the colors you selected earlier.

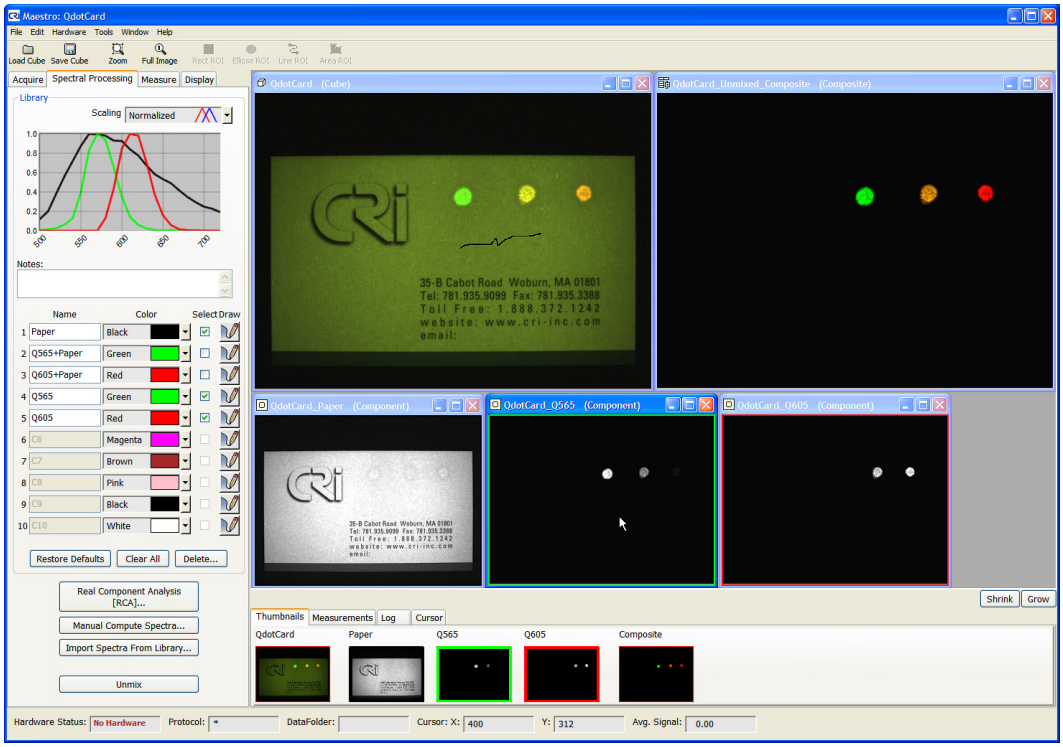


Figure 25. Grayscale and Composite Images

4. Save this protocol in case you ever want to acquire and unmix this (or a similar) CRi business card with QDot spots again in the future. Select **Save Protocol** from the **File** menu. Select a location and enter a name such as *"qdotcard"* for this example. As discussed in "Chapter 4, Method Development," protocols save cube acquisition settings, the current spectral library, and the current hardware settings so you don't have to set them again each time you unmix or analyze a similar specimen.

5. Now you are ready to quantify the results by taking measurements of the unmixed QDot spots in the component images.

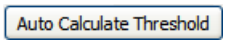
Quantifying Results

After unmixing the cube, use the controls on the **Measure** panel to quantify the results by measuring the QDot spot regions. This section provides specific step-by-step instructions for measuring the QDot spots. For more detailed information on quantifying results, see "Chapter 7, Quantifying Results."

To measure the QDot regions:

1. With the unmixed cube displayed, as shown in Figure 25, switch to the **Measure** panel.
2. Click the **qdotcard_Q565** component image to make it active.
3. In the **Millimeters Per Pixel** box, make sure **Stage Height** is set to 1B.
4. In the **Measure Objects** box, **Min. Connected Pixels** should be 100 and the **Draw Region Labels** check box should be selected.

5. Click the **Auto Calculate Threshold** button in the **Threshold Segmentation** box.



6. The Maestro software automatically creates measurement regions around the two QDot spots in the image.

Tip: You can use the **Zoom** tool to draw a zoom area around the regions to view them more clearly.

7. Repeat the procedure to find the measurement regions around the QDot spots in the **qdotcard_Q605** component image. Your screen should look similar to Figure 26.

Tip: If you prefer to draw the regions manually, click the **Draw** button to draw regions. (See “Manually Drawing and Modifying Regions” on page 79.)

8. You can adjust the transparency level and the color of the threshold mask using the **Transparency** slider and color drop down box.

9. Notice that the **Measurements** page at the bottom of the screen displays the measurements of the regions in the active image (the Q565 image in Figure 26). The **Total Signal** column shows each region’s relative measure of fluorescence. This column and the **Area (Pixels)** column are useful for comparing the amount of fluorescence in each region.

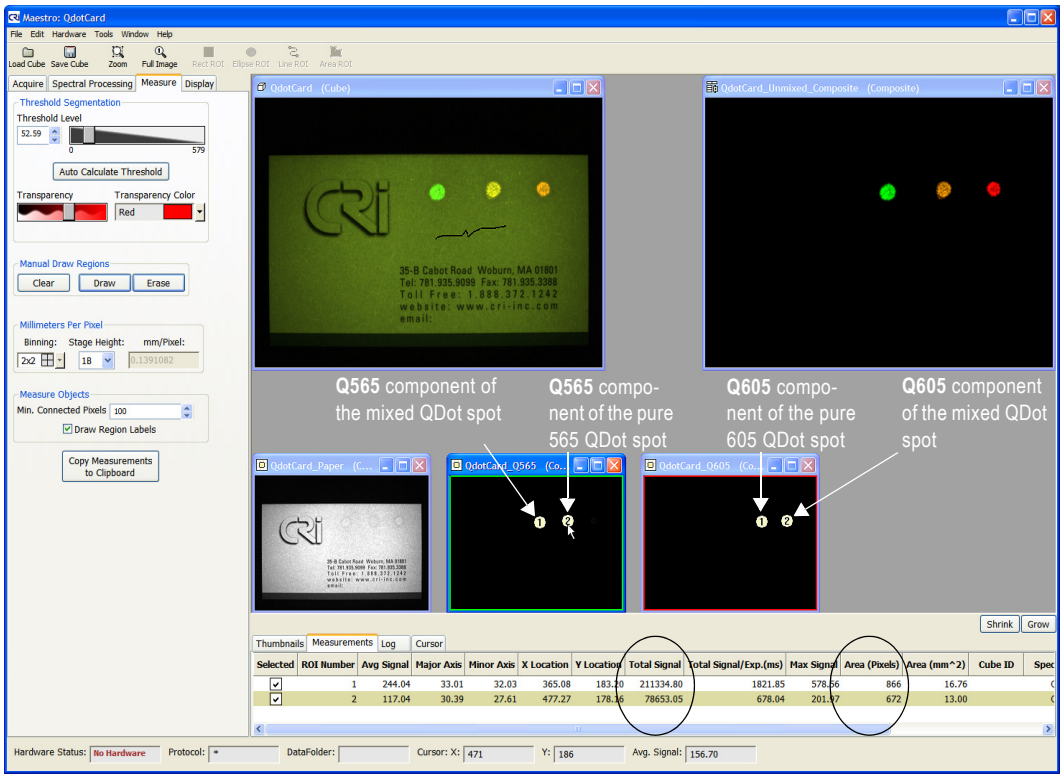

Maestro: QdotCard
Auto Calculate Threshold
Q565 component of the mixed QDot spot
Q565 component of the pure 565 QDot spot
Q605 component of the pure 605 QDot spot
Q605 component of the mixed QDot spot

Figure 26. Measured QDot Spots

Chapter 4

Method Development

Method development is a fundamental component of any good experiment, and building spectral libraries is a key part of method development. This chapter discusses methods for building accurate and reliable spectral libraries. We will explain how to create and save libraries using a variety of sample specimens, and we will discuss their unique characteristics. “Chapter 6, Unmixing Spectral Images” discusses how to open libraries and use them to unmix images.

If you are new to the Maestro *in-vivo* imaging system and have not already familiarized yourself with the rest of this manual, we suggest that you do so now. Then return to this chapter for further study.

Topics in this chapter: **Page**

- Methods for Building Spectral Libraries 40
- Saving Spectral Libraries 43
- Saving Protocols 43
- Importing Spectra Into a Library 44
- Practice Exercise 45

Methods for Building Spectral Libraries

You should create a spectral library for each of your experiments or series of experiments. Acquire or open the image cube you want to use to create the library. “Chapter 5, Acquiring Spectral Images” describes in detail how to acquire image cubes of your specimens.

There are a number of ways to build a spectral library. A few examples include:

- Using the **Real Component Analysis (RCA)** feature to automatically compute and unmix spectra (see “Computing and Unmixing Spectra Automatically” on page 65 for detailed instructions). This process adds spectra to the library, which you can modify as needed and then save.
- Using the **Manual Compute Spectra** feature, which lets you specifically sample spectral signatures from images and save them in a library (see “Computing and Unmixing Spectra Manually” on page 67 for detailed instructions). If you completed the exercise in Chapter 3, then you are already familiar with manually computing pure spectra.
- Adding spectra from other libraries, component images, or composite images of the same or similar specimens (see “Importing Spectra Into a Library” on page 44).
- Also, depending on the nature of your experiment and the types of dyes or fluorophores used, you may have imaged a single animal or two animals from which you will obtain the positive and negative controls. Use negative controls for the autofluorescence spectrum; use positive controls for “pure” spectrum calculations.

After building the library, save it as described in “Saving Spectral Libraries” on page 43.

Tips for Spectral Library Development

- There is no substitute for proper controls. Use negative controls and/or subjects that do not contain exogenous fluorophores for determining autofluorescence spectra. Use positive controls for determining the spectra of fluorophores of interest, using one or more animals for each fluorophore.
- The computed pure spectrum should be similar to, but not necessarily the same as, published spectra of that fluorophore. Variations in the label’s physical environment or depth within tissue can cause spectral shifts.
- Verify that your libraries work correctly by testing them on a few positive and negative controls. Use critical judgement.
- Unmix into white or pink for autofluorescence. Unmix into red, green and/or blue for fluorophores of interest. This will give you a nice outline of the mouse with colors superimposed.
- If you want to make a fluorophore or autofluorescence “disappear,” assign it to the black channel.
- Make sure you save the library for later use.

Obtaining Autofluorescence and Label Spectra from a Single Animal

In the following example (sample courtesy of Dr. Shuming Nie, Emory University), we use **Manual Compute Spectra**. We obtain the autofluorescence signal of the mouse from an area of pure autofluorescence. We obtain the mixed 630 nm quantum dot fluorescence plus autofluorescence (yellow spectrum, not shown) from the area of the tumor. In this instance, we are able to use a single mouse because the dye is limited to the immediate area of the tumor. The red 630 nm dye spectrum was computed using the white "autofluore" spectrum and the yellow "mixed" spectrum.

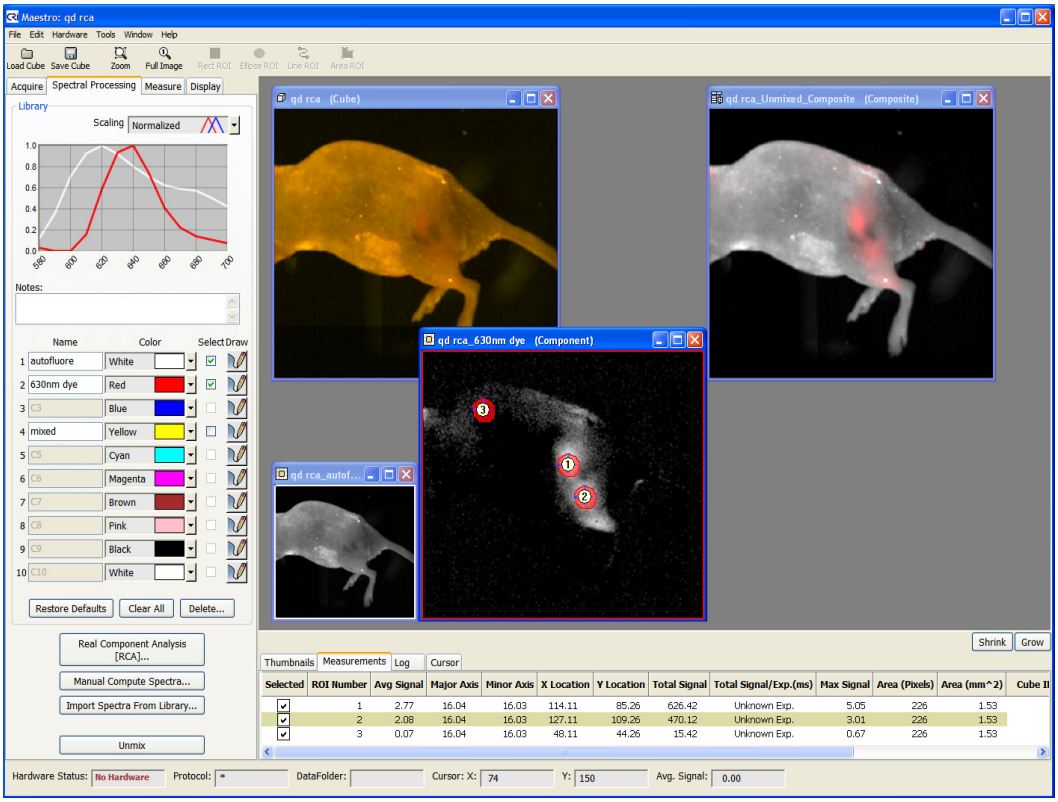


Figure 27. Autofluorescence and Label Spectra from a Single Sample

Notes:

- In this example, the autofluorescence signal is named "autofluore" and the fluorescence signal is named "630 nm dye."
- After unmixing this cube, we zoom in on the **qd rca_630nm dye** component image and use the manual **Draw** tool on the **Measure** panel to draw a measurement region around the tumor. We then copy the region (right-click on the region and select **Clone Region**) and move the copies to other locations within the image to compare fluorescence signals.
- As you can see from the **Measurements** data page at the bottom of the screen, the area of the tumor (Region #1), where specific binding has occurred, fluoresces the brightest with a **Total Signal** of 626 counts. Region #2, which represents a dimmer

region of q-dot binding in the tumor, also fluoresces, but not as brightly, with 470 counts. Region #3 (away from the tumors) shows almost no fluorescence with only 15 counts.

Obtaining Autofluorescence and Label Spectra from Two Animals

In this example, the upper mouse was injected with Alexa Fluor™ 680, which dispersed throughout the mouse's body. The lower mouse is an uninjected "negative" control. Due to the presence of the dye everywhere in the mouse, the use of an uninjected mouse was necessary in order to obtain a representative autofluorescence signal.

Using **Manual Compute Spectra**, we obtain the autofluorescence signal from the negative control mouse and a mixed Alexa Fluor 680 plus autofluorescence (yellow spectrum, not shown) from the area of the injected mouse that fluoresces brightly. (We ensure that the signals in the selected regions are not saturated). The red AF680 spectrum was computed using the white "autofluore" spectrum and the yellow "mixed" spectrum.

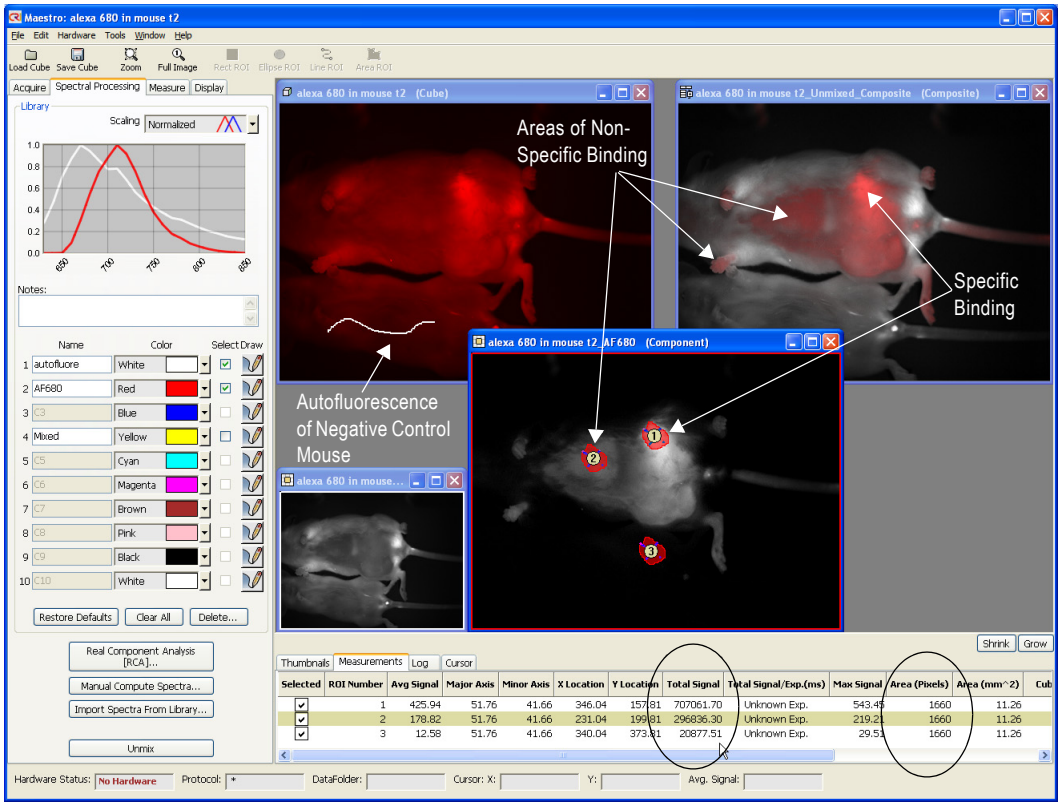


Figure 28. Autofluorescence and Label Spectra from Two Animals

Notes:

- In this example, the autofluorescence signal is named "autofluore" and the fluorescence signal is named "AF680."
- After unmixing this cube, we zoom in on the **alexa 680 in mouse t2_AF680** component image and use the manual **Draw** tool on the **Measure** panel to draw a measurement region around the tumor. We then copy the region (right-click on the region and

select **Clone Region**) and move the copies to other locations within the image to compare fluorescence signals.

- As you can see from the **Measurements** data page at the bottom of the screen, the area of the tumor (Region #1), where specific binding has occurred, fluoresces the brightest with a **Total Signal** of 707,061. (This is the region's relative measure of fluorescence.) Region #2 represents an area of non-specific binding and exhibits a bright fluorescence signal as well. Region #3 represents the signal from a mouse with no added fluorophore. Notice the much lower relative measure of fluorescence.

Saving Spectral Libraries

By saving your spectral libraries, you make them available for use again later when performing analyses on similar specimens. Instead of just saving the library, you could also save the Maestro protocol, which saves all of the Maestro acquisition and analysis settings (see "Saving Protocols" on page 43 for how to save protocols.)

To save the current spectral library:

1. Select **Save Spectral Library** from the **File** menu. The **Choose Spectral Library** dialog box opens.
2. Navigate to the folder where you want to save the spectral library, then type a name in the **File Name** field.
3. Make sure "Spectral Libraries" is selected in the **Save As Type** box and click the **Save** button.

If you want to save the library as text files, then select the "Text Files" option instead. This format cannot save color or hardware settings, but it can be opened in other applications. For example, these text files could be opened in Microsoft® Excel® for graphing, etc.

Saving Protocols

The Maestro software saves its settings in "protocols," which are recipes for collecting and measuring data. Protocols contain the following information:

- acquisition settings including region of interest and binning, wavelength settings, filter selections, exposure times, and hardware settings (bit depth, stage height, etc.)
- associated spectral library
- auto save options
- measurements options including threshold level and minimum connected pixels

Whenever you make changes to any hardware settings or acquisition settings, or edit the current spectral library, save those settings in the current protocol or create a new one. By saving your protocols, you make them available for use again later when acquiring image cubes of similar specimens.

To save a protocol:

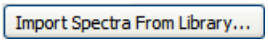
1. Select **Save Protocol** from the **File** menu.
2. In the **Choose Protocol** dialog box, select the name of an existing protocol if you want to overwrite it, or enter a new name in the **File Name** field.
3. Click **Save** to save the protocol.

Note: When you exit the Maestro application, you will be prompted to save the current protocol if you have changed any hardware or software settings during the current session. Be sure the save the protocol if you want to use the new settings again.

Importing Spectra Into a Library

From an Existing Library

You can import individual spectra from other libraries.

1. Press the **Import Spectra From Library** button on the **Spectral Processing** panel. 

2. The **Choose Spectral Library** dialog box will prompt for the file name of a spectral library. Spectral libraries have a .csl file name extension. Spectral libraries from older versions of the software with *.txt file extensions may also be opened.
3. Double-click the name of the library to open it.
4. The **Import Spectra From Library** dialog box opens, which lists all the spectra stored in that library.
5. Make sure there is a check-mark in the **Select** column next to each spectrum you want to import, then click the **Transfer to Library** button.
6. If you want to load spectra from another library, click the **Load** button and select a library to load.
7. Click **Close** when finished importing spectra.

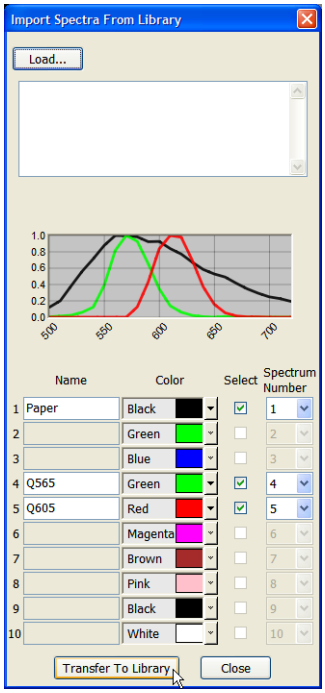


Figure 29. Import Spectra

From a Saved Component Image

You can import spectra from component images.

1. Open the Result Set (.umx) file that includes the component image from which you want to import spectra.
2. Right-click on the component image and select **Import Spectra** from the pop-up menu.

3. The **Import Spectra From Library** dialog box (Figure 29) opens with the spectrum used to unmix the cube into that component.
4. Make sure there is a check-mark in the **Select** column next to each spectrum you want to import, then click the **Transfer to Library** button.
5. If you want to load spectra from another library, click the **Load** button and select a library to load.
6. Click **Close** when finished importing spectra.

From a Saved Composite Image

You can import spectra from composite images.

1. Open the Result Set (.umx) file that includes the composite image from which you want to import spectra.
2. Right-click on the composite image and select **Import Spectra** from the pop-up menu.
3. The **Import Spectra From Library** dialog box (Figure 29) opens with all the spectra used to unmix the cube.
4. Make sure there is a check-mark in the **Select** column next to each spectrum you want to import, then click the **Transfer to Library** button.
5. If you want to load spectra from another library, click the **Load** button and select a library to load.
6. Click **Close** when finished importing spectra.

Practice Exercise

In this exercise you will open and analyze an image cube representing a mouse with a quantum dot-labeled tumor. This exercise represents the common problem of a tumor signal that is difficult to see due to the presence of autofluorescence. We will resolve this problem using Maestro multispectral imaging. (The sample is courtesy of Dr. Shuming Nie, Emory University.)

1. To open the sample image cube of the mouse with the quantum dot-labeled tumor, click the **Load Cube** button and navigate to the sample data folder: *"C:\Maestro Data\Images\ Sample Data\Q dot mouse\Q dot mouse stack"*.
2. Select any one of the TIFF files and click the **Open** button to open the image cube.

Opening the *"Q dot mouse stack"* image cube reveals a mouse with a dimly labeled tumor (you may be able to see the quantum dot glow in the left animal, near its intestines) and relatively abundant autofluorescence.

Note: In the image, the mouse on the left was injected with an antibody labeled with a 630 nm quantum dot. The mouse on the right received no injection.

3. Use RCA to unmix the cube:

   a. Click the **Real Component Analysis (RCA)** button on the **Spectral Processing** panel.

   b. Click the **Sample Spectrum** button and use the mouse pointer to sample the autofluorescence of the right (negative control) mouse. The **Use selected background spectrum** check box is now checked.

   c. Click the **Find Component Images** button.

   d. In the **Component Images** box, click the upper left image twice to select it as the background and set its pseudo color to white.

   e. Click once on the image below the background image, which shows the quantum dot-labeled tumor, and set its pseudo color to red. Its name will default to "Signal_3."

   f. The image to the right of the red quantum dot image shows the food autofluorescence in the non-fasted control mouse. Click once on this image to obtain its spectrum. Its name will default to "Signal_4."

   g. Click the **Find Spectra** button. The computed spectra will display on the graph and appear in the color palette. If desired, rename "Signal_3" to something more descriptive like, "q-dot signal." Rename "Signal_4" to "food signal" or something similar. Your screen should look similar to Figure 30.

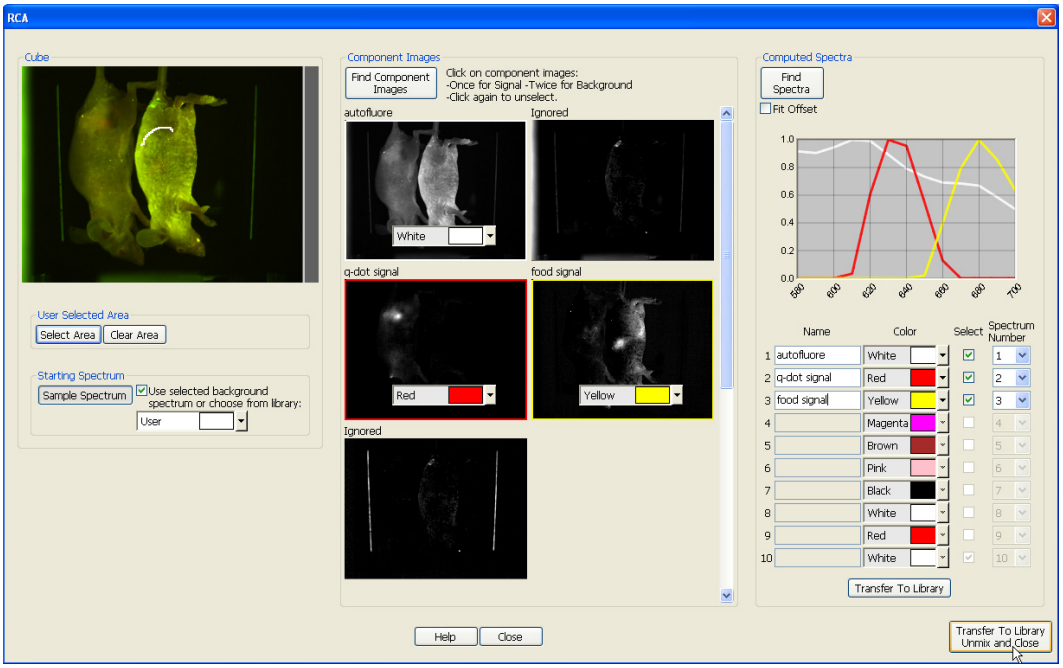


Figure 30. Unmixing with Real Component Analysis (RCA)

   h. Click **Transfer to Library, Unmix and Close**.

   i. The Maestro software unmixes the cube and displays the component and composite images in the image viewing area (Figure 31).

4. The spectral signals in the cube are now unmixed. Switch to the **Measure** panel and use the region drawing tools to quantify the signal strength of the quantum dot-labeled tumor vs. areas where non-specific binding has occurred.

Note: In this example, the mouse on the left was injected with a quantum-dot-labeled antibody[2] and was fasted for three days prior to imaging. By contrast, the mouse on the right was not injected with the fluorescent antibody and was not fasted prior to imaging. The thigh of the left mouse exhibits specific binding of the antibody (region #1); the rest of the skin of the mouse exhibits non-specific quantum dot fluorescence. The right mouse exhibits no quantum dot fluorescence. Conversely, the left mouse exhibits no food-related autofluorescence signal due to having been fasted, while the right mouse exhibits food autofluorescence in the region of its stomach.

   a. Switch to the **Measure** panel and click the **Draw** button.

   b. Draw a region around the tumor, which represents specific binding of the label (shown as region #1 in Figure 31). (Refer to "Manually Drawing and Modifying Regions" on page 79 for help drawing regions.)

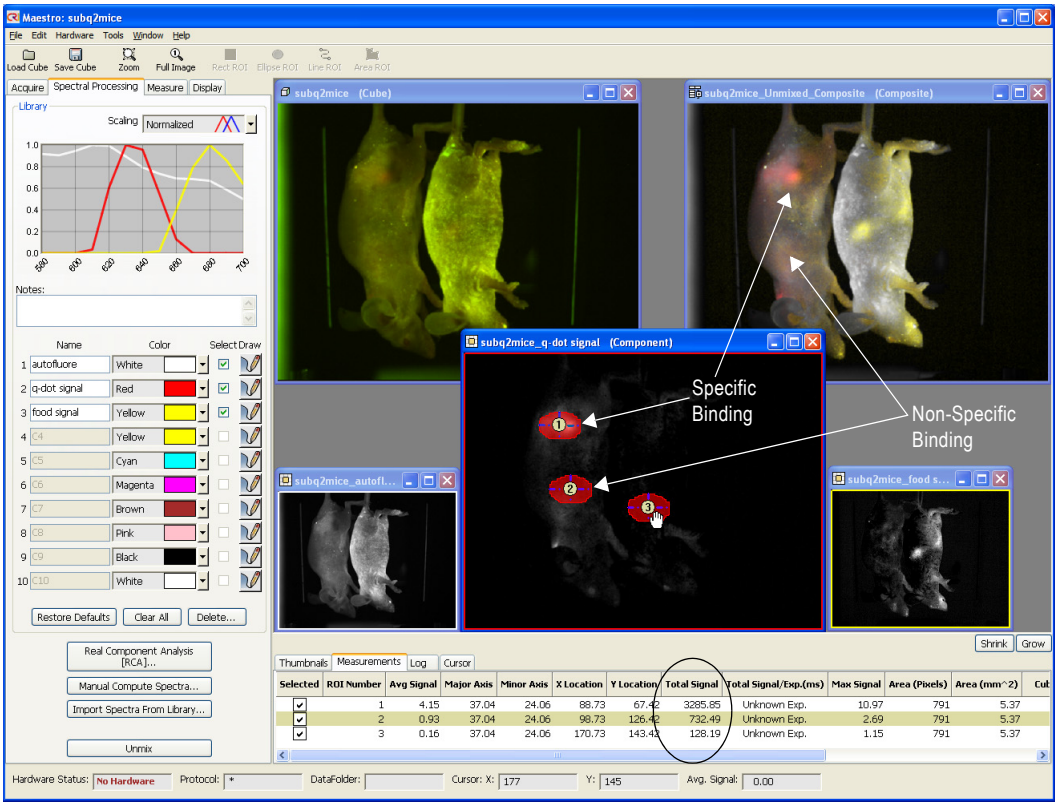

Maestro: subq2mice
File Edit Hardware Tools Window Help
Load Cube Save Cube Zoom Full Image Rect ROI Ellipse ROI Line ROI Area ROI
Acquire Spectral Processing Measure Display
Library
Scaling Normalized
Notes:
Name Color Select Draw
1 autofluore White
2 q-dot signal Red
3 food signal Yellow
4 C4 Yellow
5 C5 Cyan
6 C6 Magenta
7 C7 Brown
8 C8 Pink
9 C9 Black
10 C10 White
Restore Defaults Clear All Delete...
Real Component Analysis [RCA]...
Manual Compute Spectra...
Import Spectra From Library...
Unmix
subq2mice (Cube)
subq2mice_Unmixed_Composite (Composite)
subq2mice_q-dot signal (Component)
Specific Binding
Non-Specific Binding
subq2mice_autofl...
subq2mice_food s...
Shrink Grow
Thumbnails Measurements Log Cursor
Selected ROI Number Avg Signal Major Axis Minor Axis X Location Y Location Total Signal Total Signal/Exp.(ms) Max Signal Area (Pixels) Area (mm^2)
1 4.15 37.04 24.06 88.73 67.42 3285.85 Unknown Exp. 10.97 791 5.37
2 0.93 37.04 24.06 98.73 126.42 732.49 Unknown Exp. 2.69 791 5.37
3 0.16 37.04 24.06 170.73 143.42 128.19 Unknown Exp. 1.15 791 5.37
Hardware Status: No Hardware Protocol: DataFolder: Cursor: X: 177 Y: 145 Avg. Signal: 0.00

Figure 31. Images Showing Pure Unmixed Signals

2. Xiaohu Gao, Yuanyuan Cui, Richard M. Levenson, Leland W.K. Chung, Shuming Nie "In vivo cancer targeting and imaging with semiconductor quantum dots" *Nature Biotechnology*, April, 2004.

c. Right-click on the new region and select **Clone Region** from the pop-up menu. Drag the clone to another region of the same mouse to measure the strength of non-specific binding. The **Measurements** of region #2 shows that the signal strength in this region is approximately one-fourth the strength of region #1.

d. You can clone the region again and drag this clone to a region within the negative control mouse. Viewing the **Measurements** of region #3 confirms that there is no label present in the negative control mouse.

Chapter 5

Acquiring Spectral Images

Whenever possible, acquisition of image cubes should be done using previously established Maestro protocols that have been saved and validated with your Maestro *in-vivo* imaging system for each of your specimens/experiments. Protocols are recipes for collecting and measuring data. See “Tips for Obtaining Quantitative Results” and “Acquiring Images Using a Saved Maestro Protocol”. Protocols are discussed further in “Saving Protocols” on page 43.

This chapter explains how to acquire an image cube using the cube acquisition functions on the **Acquire** panel. Once you have specified the cube acquisition settings for a particular type of specimen, save the protocol for repeated use throughout the experiment with the same or similar specimens.

Topics in this chapter: **Page**

- Tips for Obtaining Quantitative Results 50
- Acquiring Images Using a Saved Maestro Protocol 50
- Viewing a Live Image Stream 51
- Selecting Camera Binning and a Region of Interest 51
- Specifying the Current Wavelength and Exposure 52
- Selecting a Filter and Cube Wavelengths 54
- Acquisition Setup Using Multiple Filters 55
- Flat Fielding 58
- Acquiring a Mono Image (Snap Shot) 58
- Acquiring an Image Cube 58
- Acquiring Timed Sequences of Image Cubes 59
- Saving Images and Image Cubes 60

Tips for Obtaining Quantitative Results

- Set up all hardware identically each time you acquire images during the course of an experiment. Use the same stage height and illuminator positions; select the same f-stop and filter set.
- Orient the mouse (or mice) similarly.
- Use one spectral library for unmixing all datasets.
- Make sure you've done control samples first!
- Save resulting images as "Unscaled Data" so they can be exported to other quantitation software, if desired.

Acquiring Images Using a Saved Maestro Protocol

If a Maestro protocol has already been saved, tested, and validated for the type of specimen/cube you want to acquire, load the protocol first, then acquire the cube using the settings from the protocol. Protocols are recipes for collecting and measuring data.

By using a saved protocol to acquire images of similar specimens throughout an experiment, you don't have to specify acquisition settings every time. This helps ensure consistency throughout the experiment. Protocols can be opened before or after loading an image cube.

Note: Be aware that using a protocol from one specimen to acquire an image of a specimen that contains different fluorophores will lead to incorrect results.

To open a protocol:

1. Select **Open Protocol** from the **File** menu.
2. If a protocol is already open, or if you have made any changes to settings that normally get saved in a protocol, Maestro will ask if you want to save the current changes.
3. In the **Choose Protocol** dialog box, select the protocol you want to open and click **Open**.

Tip: You can also open a Maestro protocol by double-clicking the protocol (*.pro) file in a My Computer directory window. If Maestro is not already open, this action will open Maestro with the selected protocol. You can also drag a protocol file and drop it into an open Maestro window to open the protocol.

4. Maestro now contains all the settings of the selected protocol.

Viewing a Live Image Stream

You can view a live grayscale image of the sample specimen:

To view a live image:

1. Click the **Live** button at the top of the **Acquire** panel. A live image of the sample specimen opens in a **Live Stream** window within the image viewing area.

   - The frame rate of the live image varies according to the specified exposure time.
   - If you see an all black image, the exposure setting may be too low. Also, make sure either the interior lights or the illumination light source is turned on and the illumination shutter is open.
   - Saturated pixels appear red in the live image. If some or all of the image is solid red, the image is saturated. The exposure setting may be too high. Make sure the door of the imaging module is closed.

2. Click the **Autoexpose Mono** button to automatically adjust the exposure time to the best setting for the current wavelength.

To freeze the live image:

Click the **Live** button a second time. The image in the **Live Stream** window remains frozen until you click the **Live** button again.

Selecting Camera Binning and a Region of Interest

Figure 32. Region Of Interest and Binning Selectors

Binning combines multiple pixels into a single pixel. Higher binning reduces exposure time and image size. However, higher binning also results in a lower resolution image. There are three camera binning options in the **Binning** selector. The default setting is 2-pixel (2x2) binning.

To set camera binning:

Select one of the following:

- If you want no binning, for maximum image resolution and size, select the 1x1 binning option.
- 2x2 binning combines each 2x2 square of pixels into one pixel, thus reducing the resolution in each direction by a factor of 2, but requiring 1/4th the exposure time.
- 4x4 binning combines each 4x4 square of pixels into one pixel, thus reducing the resolution in each direction by a factor of 4, but requiring 1/16th the exposure time.

The **ROI** (Region of Interest) selector sets the imaging area, which may be set to include all or just a portion of the entire field of view. The active area is always centered, and there are five ROI options in the **ROI** selector. Four options are predefined, and one is user-definable. The default setting is Full-frame.

To select a region of interest:

Do one of the following:

- To capture the Full-frame, 2/3-frame, 1/2-frame, or 1/3-frame, select from the predefined options.
- To select a custom region of interest, select "Custom" and use the mouse pointer to draw an ROI box within the **Live Stream** window. You can also create a custom region of interest by selecting **Set Custom Camera ROI** from the **Hardware** menu.

Specifying the Current Wavelength and Exposure

The **Wavelength and Exposure** box is used to manually adjust the current **Wavelength** in nanometers (nm) and the **Exposure** in milliseconds (ms).

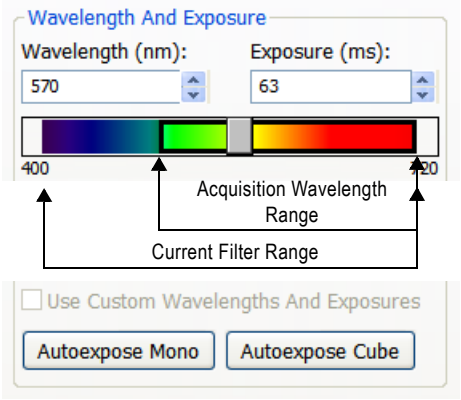


Figure 33. Wavelength and Exposure

Adjusting the Current Wavelength

Depending on the expected wavelength of the emission light, you may want to adjust the current wavelength for the filter being used.

To manually set the current wavelength:

Do one of the following:

- Drag the slider to the desired wavelength. The slider snaps to the wavelength intervals set in the acquisition settings. As shown in Figure 33, the wavelength slider indicates the current filter range. The highlighted region on the slider represents the acquisition wavelength range. The colors on the slider approximate how we would see the wavelengths within the range.

- Type a value or use the up/down scrollers in the **Wavelength** text box. When typing a value, you can set the wavelength in 1 nm increments. The up/down scrollers snap to 10 nm intervals.

Adjusting the Exposure Time

Use the autoexposure buttons to automatically determine the correct exposure setting, or enter a value (in milliseconds) in the **Exposure** entry field. You can also use the **Custom Wavelengths And Exposures** option. This option is enabled only if custom collection settings have already been specified. (See "Specifying Custom Collection Settings").

To manually enter an exposure time:

Type an exposure value into the **Exposure (ms)** text box or use the scrollers to increase or decrease the current value in increments of one millisecond.

If you are using the multiple filter feature (see "Acquisition Setup Using Multiple Filters" on page 55), you can enter a different exposure time for each filter in the sequence. Select the first filter letter ("A") in the **Multi Acq** drop down box and enter an exposure time. Repeat for each filter in the filter sequence.

To autoexpose a cube:

1. Before you acquire an image cube, click the **Autoexpose Cube** button.
2. If you are using the multiple filter feature (see "Acquisition Setup Using Multiple Filters"), the system will prompt you to install the correct filter set. Install the requested filter set before continuing.
3. Maestro will calculate the exposure time(s) that should be used to acquire an image cube of the specimen. The value will display in the **Exposure** text box.

To autoexpose at the current wavelength:

Click the **Autoexpose Mono** button. Maestro will calculate the best exposure time for the current wavelength and display the value in the **Exposure** text box.

To use custom wavelengths and exposures:

If you have specified the custom settings (see "Specifying Custom Collection Settings"), select the **Custom Wavelengths And Exposures** check box. You may now acquire the cube using the custom collection settings.

Specifying Custom Collection Settings

Custom collection settings let you use unevenly spaced acquisition settings and varying exposure times to acquire an image cube. You can specify new settings and save them, or you can load a saved wavelength and exposure table.

To specify custom collection settings:

1. Select **Edit Hardware Settings** from the **Hardware** menu (or press Ctrl+H). The **Edit Protocol** dialog box opens.

2. Click the **Custom Collection Settings** tab.
3. The **Wavelength/Exposure** table defaults to the acquisition settings of the currently selected presets.
4. To change the defaults, specify new **Start/End Wavelengths** and **Step** interval using the drop down boxes in the **Fill Wavelengths** area below the table.
5. Specify the exposure **Time (milliseconds)** in the **Fill Exposures** box.
6. Return to the **Wavelength/Exposure** table and edit individual settings as necessary.
7. Click **OK** to save the settings and close the dialog box.
8. On the Acquire panel, select the **Custom Wavelengths And Exposures** option to use the custom collection settings to acquire the image cube. (All fields in the **Cube Wavelength Selection** box become disabled.)

To save custom collection settings:

1. After specifying custom collection settings, click the **Save** button. The **Choose Custom Table** dialog box opens.
2. Specify a directory and filename and click **Save** to save the new wavelength and exposure table.

To load a wavelength and exposure table:

1. Click the **Load** button on the **Custom Collection Settings** tab. The **Choose Custom Table** dialog box opens.
2. Locate the table you want to load and click **Open**. The collection settings appear in the **Wavelength/Exposure** table. Click **OK** to use the custom settings.

Selecting a Filter and Cube Wavelengths

Filter settings define the wavelengths for which the filter is tuned when acquiring an image cube. The **Cube Wavelength Selection** box on the **Acquire** panel has preset acquisition settings as well as entry fields for manually entering cube wavelengths.

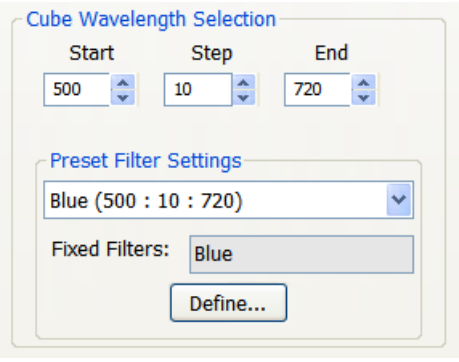


Figure 34. Cube Wavelength Selection

Using Preset Filter Settings

The **Preset Acquisition Settings** store fixed filter names with their corresponding acquisition wavelengths. For example, “Blue (500 : 10 : 720)” means these filter settings are to be used with the excitation filter and emission filter marked with a blue dot on its slider. The starting wavelength for this filter set is 500 nm, the ending wavelength is 720 nm, and images are acquired in steps of 10 nm.

To select a predefined filter set:

Select an option from the **Preset Filter Settings** drop down box. (The wavelength setting for the Live image autoadjusts to within the wavelength range of the current filter selection.)

Manually Entering Acquisition Settings

If you don’t see the filter settings you want among the **Preset Filter Settings**, you can edit the current values in the **Start**, **Step**, and **End** wavelength text boxes. When you edit any of these values, a new “Unnamed” filter setting is automatically created using the new manual settings.

Modifying and Saving Preset Filter Settings

If you don’t see the filter settings you want in the **Preset Acquisition Settings** drop down box, you can edit and save the preset filter settings for use again later:

To edit predefined acquisition settings:

1. On the **Acquire** panel, click the **Define** button below the **Preset Filter Settings** drop down box.
2. The **Edit Protocol – Filter Settings** dialog box will open.
3. Edit the settings as needed:
   - To edit existing settings, select the filter setting name in the list and edit its tunable filter settings. Click the **OK** button to save.
   - To create a new filter setting name with a new set of tunable filter settings, click the **New** button. Enter a label name and click **OK** to add the new filter setting name to the list. Then specify its tunable filter settings and click the **OK** button to save.
   - To delete a currently selected filter setting name, click the **Delete** button.
   - To restore all preset filter settings to their factory defaults, click the **Factory Defaults** button. This will also delete all new filter setting names you have saved.

Acquisition Setup Using Multiple Filters

The Maestro system offers a **Multi Filter Support** feature that lets you acquire a cube while employing two or more filter sets. For example, if you wanted to acquire an image cube that includes the acquisition wavelengths of the blue filter set (500 nm to 720 nm) as well as the red filter set (680 nm to 950 nm), then you could use this feature. The acquisition

wavelengths of the different filter sets may overlap, as with the blue and red filters, but wavelength overlap is not required.

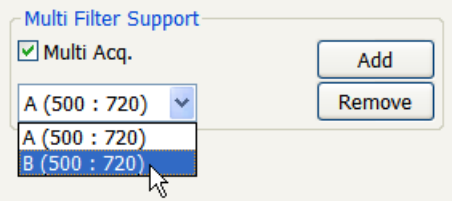
Note: There is no need to select and use the **Multi Filter Support** option when acquiring image cubes that require a single filter set.

While different exposure times can be assigned to each filter set in a multi-filter acquisition, the following parameters must be the same for all filter sets. Any changes you make to these parameters apply to all the filter sets you may have selected.

- Camera ROI
- Camera binning
- Camera bit depth
- Camera gain

To configure multiple filter sets:

1. Click the **Add** button in the **Multi Filter Support** box. This enables the **Multi Acq** check box and adds a new item ("B") to the filters drop down box.



2. "A" in the drop down box represents the first filter that will be used during cube acquisition. With "A" selected, choose the first filter set you want to use to acquire the cube.
3. Select "B" in the drop down box and choose the second filter set to use during acquisition.
4. If you want to use a third filter set, click the **Add** button again. The next letter ("C") will be added to the drop down box. Select "C" and choose the third filter set to use during acquisition. Repeat this process for each filter set you want to use.
   - If you want to remove a filter set from the list, select its corresponding letter and click the **Remove** button. The remaining filters will move up in the sequence.
   - If you want to autoexpose or acquire a cube using just one of the filter sets in your multi acquisition list, select the letter of the filter you want to use ("A," "B,", etc.) and clear the **Multi Acq** check box. The selected filter will be used. (See also "Saving Many Filter Protocols in One Maestro Protocol" on page 57.)
5. Save this protocol if it will be used again later (see "Saving Protocols" on page 43).
6. When you are ready to autoexpose or acquire the cube using multiple filters, select the **Multi Acq** check box. During multi-filter cube autoexposure and acquisition, the

Maestro system will pause during the operation, and will prompt you to install the next filter set in the sequence according to the filter line-up you created in the **Multi Acq** drop down box.

Maintaining Compatible Exposure Time Ratios

When you open a Maestro protocol that includes multiple filters and a spectral library obtained from a multi-filter cube, the ratios of the exposure times between the filter sets used for current cube acquisition and those of the saved spectral library must remain consistent. Using a library with an incompatible ratio of exposure times to unmix a new image cube may lead to erroneous results.

When using a saved multi-filter protocol to acquire a new multi-filter cube, it is recommended not to perform any additional autoexposures of the specimen or manually change the exposure times. If changes to exposure times are required, make sure the ratios remain consistent, such as 1:1, 2:1, 3:1, etc. If the exposure time ratios are different by 10% or more, the Maestro system will warn you with an error message, similar to the one shown here.

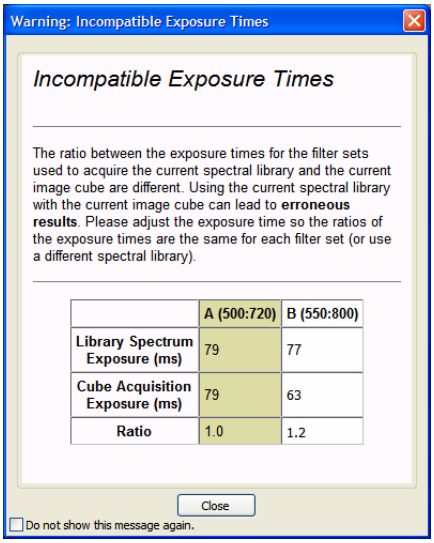

Warning: Incompatible Exposure Times
Incompatible Exposure Times
The ratio between the exposure times for the filter sets used to acquire the current spectral library and the current image cube are different. Using the current spectral library with the current image cube can lead to erroneous results. Please adjust the exposure time so the ratios of the exposure times are the same for each filter set (or use a different spectral library).
A (500:720)
B (550:800)
Library Spectrum Exposure (ms)
79
77
Cube Acquisition Exposure (ms)
79
63
Ratio
1.0
1.2
Close
Do not show this message again.

Figure 35. Incompatible Exposure Times

Saving Many Filter Protocols in One Maestro Protocol

You can use the **Multi Filter Support** feature to save multiple filter protocols in a single Maestro protocol. This is useful when you need to take multiple cubes of a specimen using different filter sets. For example, you may want to take separate cubes of the same mouse using blue, yellow, and red filter sets. (Remember, each filter set can have a different exposure time, but all sets will have the same camera ROI, binning, bit depth, and gain.)

Follow the instructions earlier in this section to configure the filter settings (blue, yellow, and red, for example) in the multi acquisition list, and then save the protocol. When you are ready to take multiple cubes of a specimen, load the saved multi-filter protocol that contains the appropriate filter sets. Choose the filter set you want to use to acquire the cube, and then clear the **Multi Acq** check box so only the selected filter set will be used.

Flat Fielding

Flat Fielding makes it possible for the Maestro software to create better, more evenly bright image cubes. When you take cubes without Flat Fielding, you may notice that the outer edges of acquired images are slightly darker than the rest of the image.

Flat Fielding creates more even image cubes by taking a reference image before acquiring the image cube. Once you have taken a reference cube, it will be saved with the current Maestro protocol, if you save the protocol. However, if you change the binning or region of interest (ROI), the current reference cube becomes invalid. You will have to take a new reference image before acquiring a cube with different binning or ROI.

1. Check the **Flat Field** check box. This activates the **Acquire Ref. Image** button. (The **Acquire Cube** button is now disabled until you acquire the reference image.)

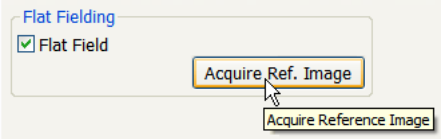


Figure 36. Flat Fielding

2. Remove the current specimen, if any, from the Maestro imaging module and replace the specimen with a stack of about ten sheets of clean white paper.
3. Click the **Acquire Ref. Image** button. (If you have activated multi-filter support, you will be prompted to install the correct filter set(s) before continuing.)
4. After acquiring the reference image, the **Acquire Cube** button is once again available. Replace the paper with your specimen, click the **Autoexpose Cube** button again, and then click the **Acquire Cube** button.

Acquiring a Mono Image (Snap Shot)

To take a snap shot at the current wavelength and exposure, click the **Acquire Mono** button at the bottom of the **Acquire** panel. This function acquires a single picture, not an image cube.

Acquiring an Image Cube

1. Autoexpose the cube as described in “Adjusting the Exposure Time” on page 53.
2. If you are satisfied with the wavelength and exposure settings, click the **Acquire Cube** button at the bottom of the **Acquire** panel to acquire the image cube.
3. If you are using the multiple filter feature (see “Acquisition Setup Using Multiple Filters” on page 55), the system will prompt you to install the correct filter set. Install the requested filter set (and each subsequent filter set) before continuing.
4. When Maestro has finished taking the image cube, a color representation of the data will display in the image viewing area.

Assigning a Sample ID to an Image Cube

Use the **Sample ID** text box on the **Acquire** panel to assign an ID to the current cube. When you save the cube, it will be saved with its sample ID. All measurements derived from the cube's component images will be saved with the cube's sample ID.

Cube IDs display in the **Cube ID** column of the **Measurements** page near the bottom of the Maestro work area.

Associating Notes with an Image Cube

Use the **Notes** text box on the **Acquire** panel to save notes with the current cube. Whenever the cube is opened, any notes previously saved with the cube will display in the **Notes** box.

Acquiring Timed Sequences of Image Cubes

The Maestro system can acquire multiple cubes of a specimen using a timed acquisition interval that you specify. This is useful for observing changes in a specimen over a specified time span.

1. Select **Acquire Time Sequence** from the **Tools** menu. The **Acquire Time Sequence** dialog box opens (Figure 37).
2. Enter the amount of time (in seconds) that you want to elapse between the **start** of each cube acquisition. For example, if you enter 60 seconds and it takes 15 seconds to acquire the first cube, there will be a 45 second lapse until the start of the next cube acquisition. Be sure to enter a time that is greater than the amount of time it will take to acquire each cube.

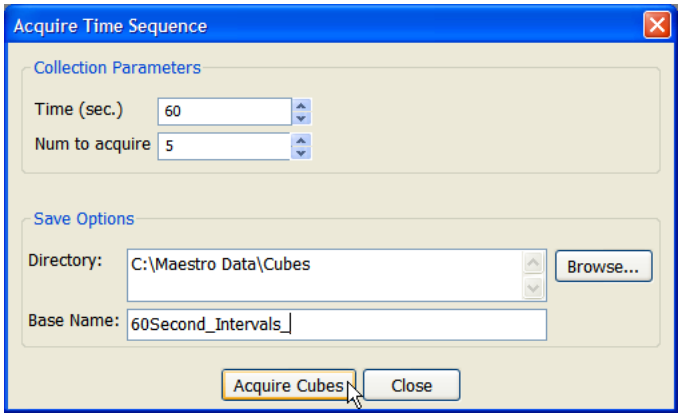


Figure 37. Acquire Time Sequence

3. Enter the number of cubes you want to acquire during this acquisition sequence.
4. Click the Browse button to select a destination directory where you want the Maestro software to save all the cubes acquired during the sequence.
5. Enter a base filename for all the saved cubes.

6. Click the **Acquire Cubes** button to begin the acquisition sequence. A progress indicator displays the amount of time until the next exposure (cube), the current exposure (cube) number, and the total time remaining until the acquisition sequence is complete.

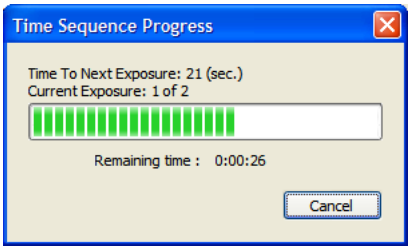


Figure 38. Timed Sequence Progress

7. The Maestro software automatically saves each cube in the specified directory.

8. When the acquisition sequence is complete, you can locate the cubes at the destination directory and open them in Maestro. As seen in Figure 39, the cube filenames begin with the base name you specified and include the date and time of acquisition.

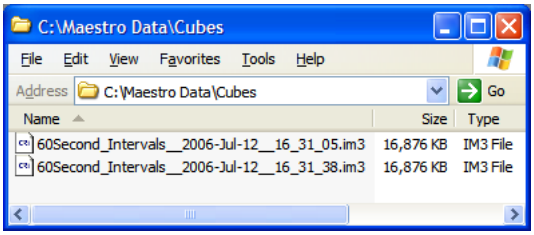
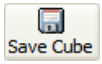


Figure 39. Cubes acquired during timed acquisition

Saving Images and Image Cubes

Tip: Devise a consistent naming convention for your images and image cubes. Use file and folder names that incorporate specimen names as well as dates and/or times and acquisition parameters such as filter cube, binning and exposure.

To save an image cube:



1. To save the current image(s) and associated data, click the **Save Cube** button on the toolbar, or select **Save Cube** from the **File** menu. The **Choose Cube** dialog box opens.
2. Navigate to where you want to save the cube.
3. In the **Save as type** box, select a cube type option:
   - Select “Image Cubes” if you want to save the cube as a single CRi image cube file. A CRi cube can include hardware settings, spectral libraries, user comments, and display settings. Cubes saved in the CRi format cannot be opened in third-party tools.
   - Select “TIFF Cubes” if you want to save the cube as a series of TIFF images. Maestro saves a TIFF image in the specified location for each of the wavelengths used to acquire the cube. When saving a cube that was acquired using multiple filters, filename_A_* will be used for the first filter set, filename_B_* will be used for the second filter set, and so on. TIFF Cubes contain only images; none of the associated data or settings is saved with this format.
4. Enter a file name for the cube and click the **Save** button.

To save images:

1. Select **Save Image** from the **File** menu and select a save option to save the image(s):
   - **Save (As Displayed)** prompts you to enter a name for the currently selected image, and saves the image as a TIFF or JPEG image.
   - **Save All (As Displayed)** prompts you to enter a name for each of the current images in the image viewing area, and saves the images as TIFF or JPEG images.
   - **Save Image (As Unscaled Data)** prompts you to enter a name for the currently selected component image, and saves unscaled image data as a TIFF component.
   - **Save All Images (As Unscaled Data)** prompts you to enter a name for each of the component images, and saves all unscaled image data as TIFF components.
2. When the dialog box opens, navigate to where you want to save the image(s) (the default location is in *"C:\Maestro Data"*).
3. Enter a file name and click the **Save** button.

Saving Image Cubes Automatically

The Maestro system can save your image cubes automatically.

To specify automatic save options:

1. Select **Edit Hardware Settings** from the **Hardware** menu, or press Ctrl+H.
2. In the **Edit Protocol** dialog box, click the **Autosave Options** tab.
3. Select the **Autosave Image Cubes** option to enable the autosave fields.
4. To specify a default directory, click the **Browse** button and select from the browse dialog box.
5. Type a base name in the **Base Name** field, if desired.
6. In the **Auto-naming Options** box, specify a naming option. A sample appears in the **File Name** field.
7. Click the **OK** button to save the changes and close the dialog box.

Viewing Cube Information

Cube information includes its size in pixels, the number of planes in the cube, a timestamp, any comments associated with the cube, and the wavelengths and exposure times used to generate the cube.

To view cube info:

Right-click on the image in the **Cube** window and select **Cube Info** from the pop-up menu. The **Cube Info** window opens.

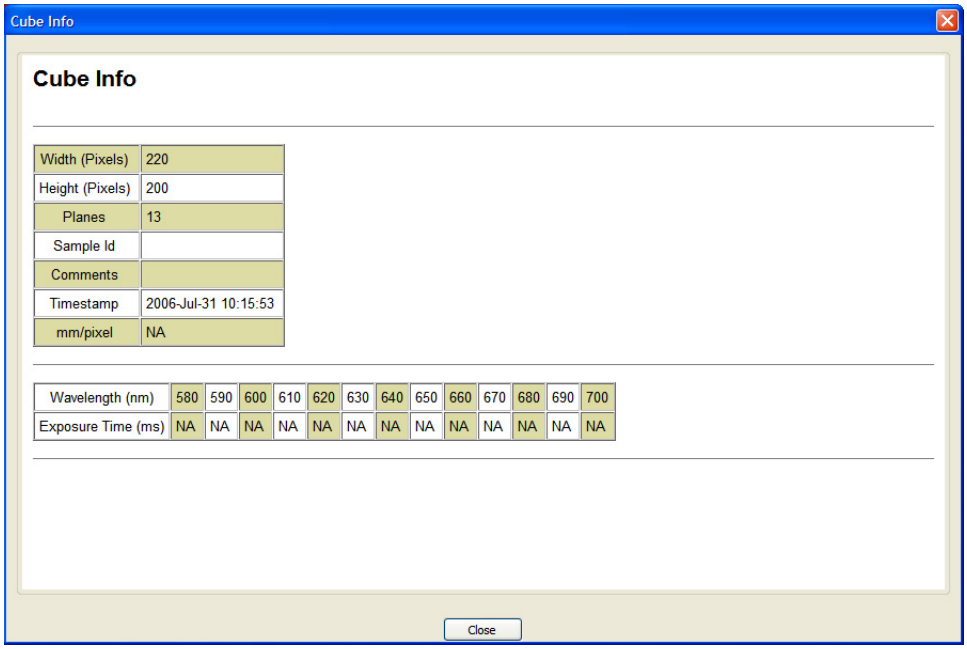

Cube Info
Cube Info
Width (Pixels) 220
Height (Pixels) 200
Planes 13
Sample Id
Comments
Timestamp 2006-Jul-31 10:15:53
mm/pixel NA
Wavelength (nm) 580 590 600 610 620 630 640 650 660 670 680 690 700
Exposure Time (ms) NA NA NA NA NA NA NA NA NA NA NA NA NA
Close

Figure 40. Cube Information

Extracting an Image Plane from a Cube

You can extract and view individual wavelength images from a cube.

1. Right-click on the image in the **Cube** window and select **Extract Channel** from the pop-up menu.
2. Select the channel you want to extract by clicking a wavelength value in the list. A monochrome image of the selected wavelength opens in a new window.

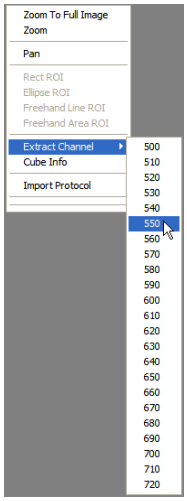


Figure 41.Extract Channel

Chapter 6

Unmixing Spectral Images

Whenever possible, spectral processing—or unmixing—of image cubes should be done using previously established Maestro protocols that you have saved and validated with your Maestro *in-vivo* imaging system for each of your specimen/experiment types. In most cases, these protocols contain spectral libraries that will be used when unmixing images (as explained in "Chapter 4, Method Development").

This chapter explains how to create a spectral library and unmix an image cube using the spectral processing functions on the **Spectral Processing** panel. Once you have created your spectral library, save the protocol and spectral library for repeated use throughout the experiment with the same or similar specimens.

Topics in this chapter: **Page**

- Unmixing with a Saved Spectral Library 64
- Opening an Image Cube 64
- Computing and Unmixing Spectra Automatically 65
- Computing and Unmixing Spectra Manually 67
- Saving An Unmixed Result Set 69
- Working With Saved Result Sets 71
- Baseline Subtraction 71
- Batch Data Processing 72
- Using Line Profiles to Analyze Signals 74

Unmixing with a Saved Spectral Library

If a spectral library has already been saved and validated for the type of specimen you want to unmix, use that library to unmix the cube. Or if an appropriate Maestro protocol is available, which should include the spectral library, then load the protocol instead (see “Acquiring Images Using a Saved Maestro Protocol” on page 50).

By using a saved protocol and/or spectral library on similar specimens to unmix and perform analyses throughout an experiment, you don’t have to recreate the spectral library every time. This helps ensure consistency throughout the experiment. Protocols and spectral libraries can be opened before or after loading an image cube.

Note: Be aware that using a spectral library from one specimen on a specimen that contains different fluorophores will lead to incorrect results.

To open a spectral library:

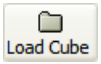
1. Select **Open Spectral Library** from the **File** menu. The **Choose Spectral Library** dialog box opens.
2. Select the location and filename of the library (or protocol) and click **Open**.

Tip: You can also open a Maestro spectral library by double-clicking the library (*.csl) file in a My Computer directory window. If Maestro is not already open, this action will open Maestro with the selected library. Or you can drag and drop the library file into an open Maestro window to open the library.

3. The color palette on the **Spectral Processing** panel will be populated with the parameters of the new spectral library. (If you opened a protocol, all of the parameters saved in the protocol take effect.)

Opening an Image Cube

1. Click the **Load Cube** button on the toolbar, or select **Open Cube** from the **File** menu.



2. In the **Choose Cube** dialog box, browse to the location where the image cube is saved.
   - If opening a CRi format (.im3) image cube, double-click the file to open it.
   - If opening a TIFF image cube, open the folder containing the images and double-click any one of the TIFF image files to open the cube.
3. The cube will open, and a color representation of it opens in the Maestro work area.

Tip: You can also open an image cube by double-clicking the cube (*.im3) file in a My Computer directory window. If Maestro is not already open, this action will open Maestro with the selected cube. Or you can drag and drop the cube (or multiple cubes) into an open Maestro window to open the cube(s).

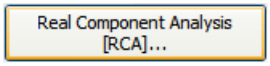
Computing and Unmixing Spectra Automatically

The **Real Component Analysis** feature lets you do the following:

- Explore how many different spectral features are present.
- Extract spectra for further analysis.
- Calculate “pure” spectra even if autofluorescence is present.

To compute spectra using real component analysis:

1. Open or acquire a cube.
2. Select the **Spectral Processing** tab.
3. Click the **Real Component Analysis** [RCA]... button.



4. The **Real Component Analysis** dialog box opens.

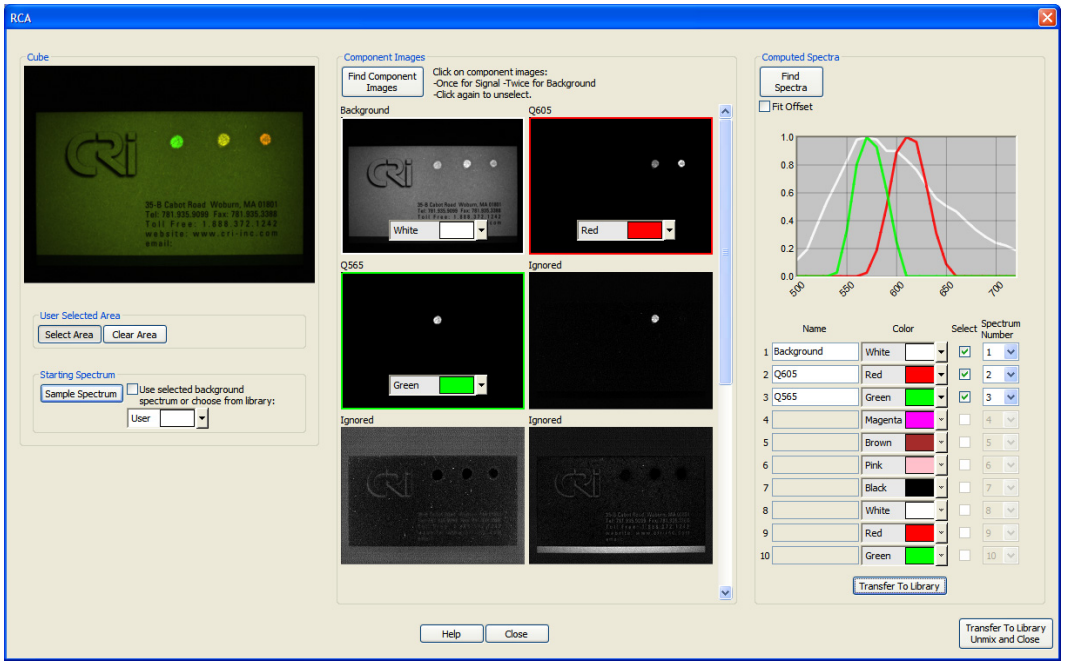


Figure 42. Real Component Analysis

5. If you want to select a smaller region of the image on which to perform the RCA computation, click the **Select Area** button and use the mouse to draw a rectangular region on the Cube image. If you don’t draw a region, the whole image will be used. Clicking the **Clear Area** button clears any user selected area from the image.
6. If you want to provide an initial guess of the background (autofluorescence) signal, click the **Sample Spectrum** button and use the mouse pointer to specify the background signal in the image cube.

- Alternately, you can import a known autofluorescence spectrum from the current spectral library by selecting from the **Starting Spectrum** drop down box. Use this option if there is no region in the current sample that contains only autofluorescence, as is often the case when using an injected fluorophore.
- These settings can be discarded by un-checking the **Use Selected Background** box.

7. Click the **Find Component Images** button in the **Component Images** box. A set of monochrome images of spectrally distinct signals will soon appear to the right of the cube display window.
8. Select component images for use in unmixing.
   - Look for images that appear to contain “significant” signals (a sample-specific judgement). To select an image, click it once. A drop down box opens and lets you accept or change the default color of the spectral library member.
   - If autofluorescence (or other background signal) is thought to be present, the image (typically the top-left) should be designated as Background by clicking twice on it. Only one image can be designated as Background.
   - Cycle through the choices by clicking once for Signal, twice for Background, and three times for Ignored.
9. Click the **Find Spectra** button in the **Computed Spectra** box.
   - If no Background component is selected, then the “purest” spectra present in the cube will be found.
   - If a Background component was selected, then all other signals will be used as inputs into a computation that removes the presence of the background spectrum to give the spectrally “pure” components.
10. Occasionally, for weak signals, there may be too much baseline offset in the data. If you did not get the desired unmixing results, select the **Fit Offset** option and see if the unmixing results improve.
11. You may now use the controls in the **Computed Spectra** box to change a spectrum's name, color, and where it should reside in the spectral library.
12. When finished, click the **Transfer to Library** button to add the spectra to the main spectral library. If a library is open already, you may be asked if you want to overwrite the existing spectra.
13. Click the **Close** button to close the **RCA** dialog box.
14. When you are ready to unmix the image, first verify that the spectra you want to unmix is selected in the library, then click the **Unmix** button.

    Alternately, you may press the **Transfer to Library, Unmix and Close** button to perform all these actions with a single click.
15. If the spectral library you have created will be used again for future unmixing operations, save the protocol and/or spectral library.

Computing and Unmixing Spectra Manually

Sampling Spectra from a Cube

If you want to compute pure spectra without using RCA, you need to sample the known and mixed spectra manually. Use the color palette on the **Spectral Processing** panel to build your spectral library by selecting colors for separating mixed signals into pure signals.

If the cube you want to unmix was acquired using multiple filters with overlapping acquisition wavelengths (see “Acquisition Setup Using Multiple Filters” on page 55), you will notice that there are two or more overlapping curves in the spectral graph for each of the spectra in cube. Although this may look unusual, the overlapping curves are used together as one spectrum for all unmixing.

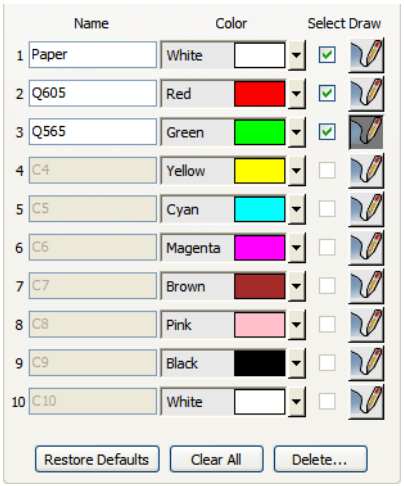


Figure 43. Color Palette

There are ten rows available that you can use to build a spectral library.

To sample spectra:

1. Open or acquire a cube and select the **Spectral Processing** tab.
2. Click the **Draw** button within the row where you want to place the sampled spectrum.
3. Click and draw on the part of the cube you want to sample. (Holding down the Control or Shift key will add additional regions to the sample.) When you release the mouse button, a check-mark appears in the **Select** column.

Tip: The Control and Shift keys also let you sample multiple cubes: Sample the current cube, then select another cube from the thumbnails and hold down the Shift or Control key while sampling to obtain an average of the spectra from the cubes.)

4. The default line sampling tool can be changed by selecting another region of interest shape from the toolbar (see “Selecting a Region of Interest Shape” on page 68).
5. Assign a helpful name to each spectrum by entering a name in the **Name** column.
6. To change the color assigned to a spectrum, select from its drop down color menu.

Selecting a Region of Interest Shape

1. Click the **Draw** button for the row where you want to place the sampled spectrum.
2. Click the button for the shape you want to use on the toolbar.

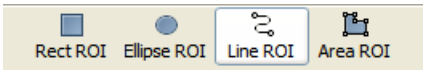


Figure 44. ROI Shapes

3. Click and drag over the part of the cube you want to sample.

Manually Computing Spectra

1. Open or acquire a cube and select the **Spectral Processing** tab.
2. After loading or building a spectral library, press the **Manual Compute Spectra** button to display the dialog box.
3. In the **Known Spectrum** box select the known spectrum—the spectrum of the autofluorescence. Multiple known spectra may be selected by holding down the Control key while making selections.
4. In the **Mixed Spectrum** box select the spectrum that represents the selected known spectra plus any additional spectrum. For example, autofluorescence plus a label.
5. The **Computed Spectrum** will be displayed in the color specified at the bottom of the dialog box.
6. If necessary, use the **Scale** functions to fine-tune the fit adjustment. See "Using Fit Offset" and "Error Scaling When Manually Computing Spectra" on page 69 for instructions.

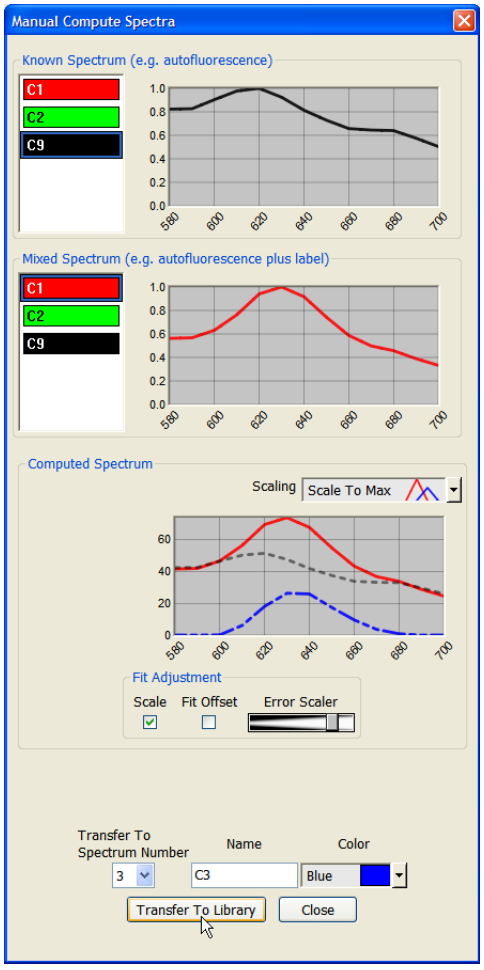


Figure 45. Manual Compute Spectra

7. Select the row, name, and color for this spectrum and press the **Transfer To Library** button.
8. Repeat this process for the other mixed spectra.
9. When you are ready to unmix the cube, close this dialog box and click the **Unmix** button on the **Spectral Processing** tab.
10. Save the new spectral library as described in "Saving Spectral Libraries" on page 43.

Error Scaling When Manually Computing Spectra

To obtain an accurate pure spectrum (which is required for an accurate unmixing), the correct amount of autofluorescence must be subtracted from the mixed spectrum. To do this, it is important that the non-overlapping regions between the "pure" fluorophore and the autofluorescence line up closely.

The Maestro software attempts to determine the amount of autofluorescence to subtract automatically. When there is a bright fluorophore signal (relative to the autofluorescence), scaling is usually not needed. In that case, clear the **Scaling** box so that Maestro will subtract the known spectra from the mixed spectra without any scaling.

When there is a weaker signal, you can manually adjust the amount of autofluorescence that will get subtracted to ensure that the best "pure" spectrum is found. This is done by checking the **Scaling** check box and sliding the **Error Scaler**.

1. In the **Computed Spectrum** box (see Figure 45), select "Scale To Max" in the **Scaling** drop down box. This provides the best visual representation of the spectra.
2. The mixed spectrum and computed spectrum are shown in their respective colors. The dotted "best fit" line is a scaled representation of the known autofluorescence signal (see Figure 45).
3. With the **Scaling** box checked, try sliding the **Error Scaler** to the right to increase the scaling (or to the left to decrease it) until the non-overlapping portions of the known and mixed spectra line up as closely as possible (as shown in Figure 45).
4. When you are satisfied with the computed spectrum, select a Spectrum Number, Name, and Color and click the **Transfer to Library** button.
5. When you are finished adding spectra to the library, remember to save it as described in "Saving Spectral Libraries" on page 43.

Using Fit Offset

Occasionally, for weak signals, there may be too much baseline offset in the data. If you did not get the desired unmixing results, select the **Fit Offset** option in the **Manual Compute Spectra** dialog box (Figure 45) and see if the unmixing results improve. The computed spectrum should look like a simple gaussian curve, which is the shape of accurate quantum dot spectra.

When you are satisfied with the computed spectrum, select a Spectrum Number, Name, and Color and click the **Transfer to Library** button. When you are finished adding spectra to the library, remember to save it as described in "Saving Spectral Libraries" on page 43.

This feature is also available when using Real Component Analysis, as explained in "Computing and Unmixing Spectra Automatically" on page 65.

Changing the Spectral Graph Scale

There are two places where you can specify spectral graph scaling: at the top of the **Spectral Processing** panel and on the **Manual Compute Spectra** dialog box.

1. Select an option from the **Scaling** drop down box above the spectral graph:

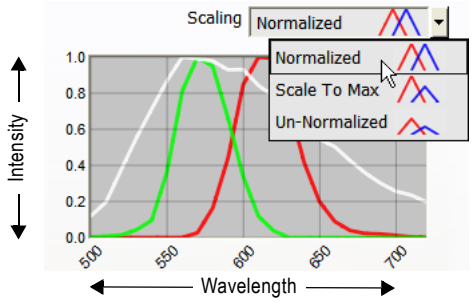


Figure 46. Spectral Graph Scaling Selector

   - Select the "Normalized" option to view the spectral curves as normalized. The Y-axis in the spectral graph is scaled 0 to 1. Each spectral curve is independently scaled to 1 at its maximum height.
   - Select the "Scale To Max" option to scale the Y-axis in the spectral graph from 0 to maximum height of the highest spectral curve in the image cube.
   - Select the "Un-Normalized" option to view the spectral curves as un-normalized. The Y-axis in the spectral graph is scaled 0 to 255 (8-bit) or 0 to 4095 (12-bit).

2. Move the cursor over the image to see how the white curve changes shape. You are seeing the spectral curve associated with each pixel as you move the mouse pointer.

Saving An Unmixed Result Set

After unmixing an image, you can save all unmixing results without having to save each unmixed image independently. The **Save Result Set** feature saves the entire workspace (excluding the cube) in a single file. This file will contain the component and composite image, the file name of the cube (but not the cube itself), an RGB representation of the cube, and other data such as measurements and regions, display parameters, user notes, and the spectral library.

You can then reload the saved result set later to review the unmixed results and conduct further analysis/quantitation. You cannot do additional unmixing, however, because there is no cube associated with a saved result set, unless you import the associated cube into the result set (see "Importing a Cube Into a Result Set" on page 71).

To save the current unmixed result set:

1. With the unmixed images on your screen, select **Save Result Set** from the **File** menu.
2. The **Choose Results Set** dialog box opens.
3. Enter a name for the new result set and click **Save**. Result set files are saved with a ".umx" file name extension.

Working With Saved Result Sets

If unmixed result sets have been saved, you can open them for further analysis and quantitation. (See “Saving An Unmixed Result Set” for instructions on saving unmixed result sets.)

To open a result set:

1. Select **Open Result Set** from the **File** menu.
2. In the **Choose Results Set** dialog box, locate and select the “result_set.umx” file you want to open, and click **Open**.

Tip: You can also open a Maestro result set by double-clicking the result set (.umx) file in a My Computer directory window. If Maestro is not already open, this action will open Maestro with the selected result set. You can also drag and drop a result set into an open Maestro window.

3. The unmixed images will open in the image viewing area. If the Maestro software can locate the cube associated with the result set (it must be a CRi format “.im3” Image Cube in its original location on the computer), the cube will open along with its unmixed images. If the Maestro software cannot find the cube or it is a TIFF Cube, an RGB representation of the cube (labeled “RGB - Cube Is Not Loaded”) will open.
4. If the cube did not load automatically, you can import the cube into the result set. See “Importing a Cube Into a Result Set” for instructions.

Importing a Cube Into a Result Set

When a result set is saved, the original image cube is not saved as part of the result set (.umx) file. An RGB representation of the cube is saved instead. When you open a result set, the Maestro software attempts to open the associated cube as well. If the associated cube cannot be found, the RGB proxy opens instead. You can replace the RGB proxy with the actual cube.

To import a cube into a result set:

1. Right-click on the RGB image and select **Import Cube** from the pop-up menu.
2. The **Choose Cube** dialog box opens.
3. Select the cube (an Image Cube or TIFF Cube) you want to import and click **Open**.

Baseline Subtraction

If there is stray light (which contributes to a baseline offset) it can be subtracted from the whole cube using Baseline Subtract. This will ensure that the background will be near zero.

1. Select **Baseline Subtract** from the **Tools** menu. The **Baseline Subtract** dialog box opens.

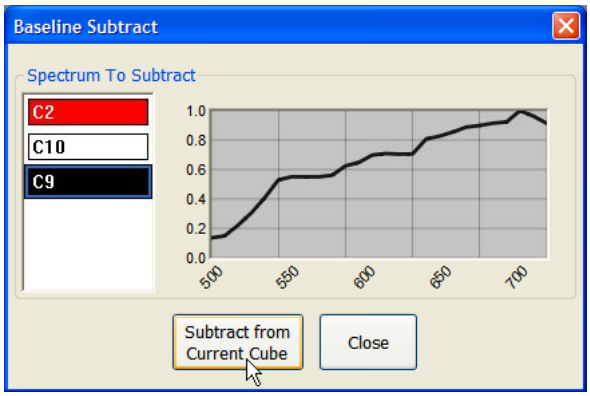


Figure 47. Baseline Subtract

2. This dialog box displays the spectra from the current spectral library.
3. Select spectra you want to subtract and click the **Subtract from Current Cube** button.
4. The Maestro software subtracts the selected spectrum from the cube and closes the dialog box. The cube's filename is also appended with "_subt" to remind you that a spectrum has been subtracted from the cube.

Batch Data Processing

In some instances, you might prefer to acquire a large number of cubes before analyzing them, or you might want to re-analyze a large set of images with new analysis settings. The Batch Data Processing feature is useful for unmixing and measuring multiple cubes or a large set of images all at once.

1. Select **Batch** from the **Tools** menu. The **Batch Data Processing** dialog box opens.

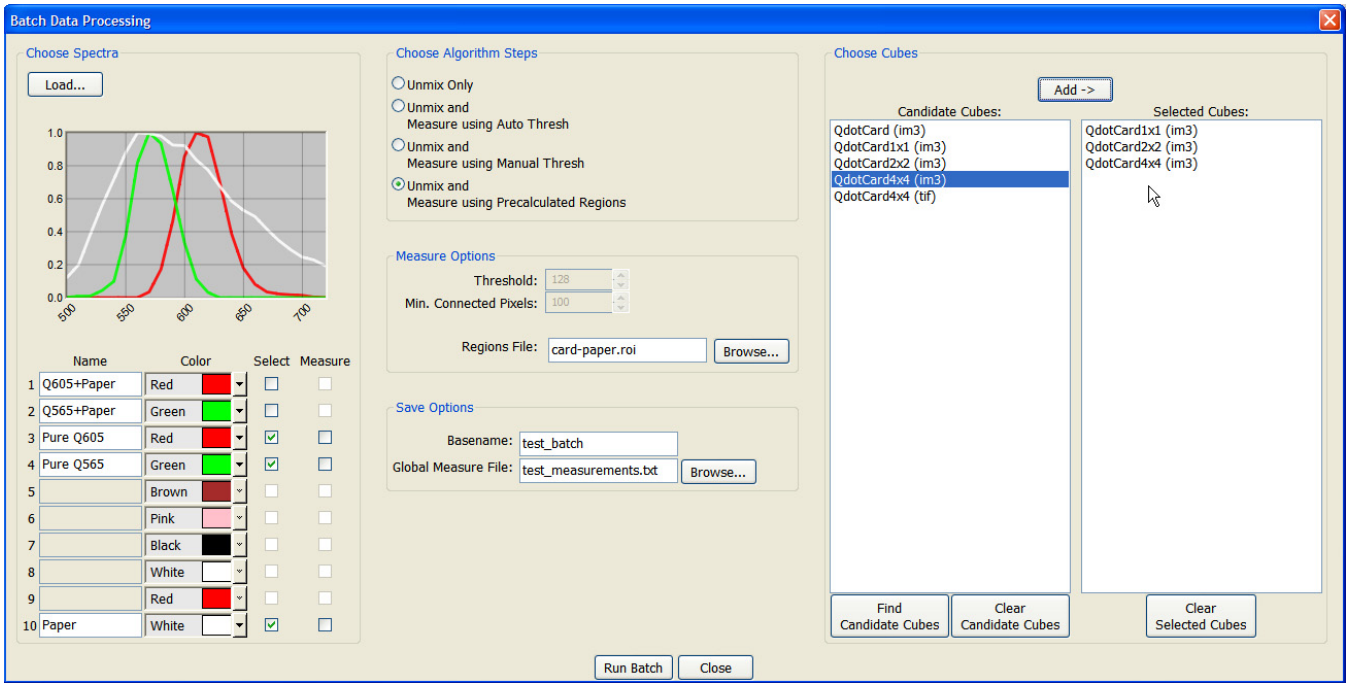


Figure 48. Batch Data Processing

2. The **Choose Spectra** box at the far left contains the current spectral library. Select the individual spectra you want to use for unmixing by checking the check box in the **Select** column. Select the component images you wish to measure by checking the control in the **Measure** column.

3. The **Choose Algorithm Steps** box selects the algorithm steps and parameters. Use the radio buttons to select one of the following options:

   - **Unmix only**
   - **Unmix and Measure using Auto Threshold**. If you select this option, then specify the **Min. Connected Pixels** parameter in the **Measure Options** box.
   - **Unmix and Measure using Manual Threshold**. If you select this option, then specify both the **Min. Connected Pixels** and **Threshold** parameters in the **Measure Options** box.
   - **Unmix and Measure using Precalculated regions**. If you select this option, then select a Regions (.roi) file that you want to use to measure the unmixed results.

4. The parameters in the **Save Options** box control how the result files are named. Results are saved in the same directory as the cube.

   - The **Basename** parameter is the file prefix. The unmixed results will be named *"basename_timestamp.umx"*. For example, if the **Basename** option is *"test_batch"*, an unmixed file would be called *"test_batch_2006-August-08_11_47_48.umx"*.

- The **Global Measure File** parameter is a file that contains all the result measurements in a single file.

5. The **Choose Cubes** box at the far right lets you select the image cubes. Click the **Find Candidate Cubes** button. A dialog box will prompt for a starting directory. The Maestro software will search this directory and the directories one level below for cubes that are compatible with the current spectral library and display them in the **Candidate Cubes** list.
6. Select from the **Candidate Cubes** list the cubes you want to process, and click the **Add** button. This will add them to the **Selected Cubes** list on the right.
7. Click the **Run Batch** button. This will iterate through the selected cubes, unmix, and measure any components selected for measurement.

Using Line Profiles to Analyze Signals

The Line Profiles tool can be used to analyze signals in component images. This tool can be used to analyze any single-channel (monochrome) image, including component images and live images in Maestro. (Cubes and composite images cannot be analyzed with the Line Profiles tool). To use this tool, you draw lines on component (or any monochrome) images, and the pixels sampled by the line show up as a plot in the Spectral Graph in the **Line Profiles** dialog box.

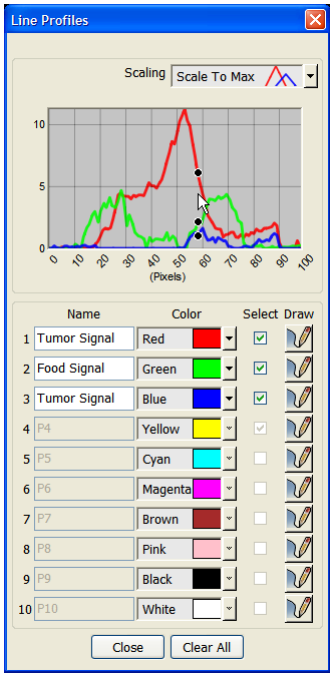


Figure 49. Line Profiles

To use the Line Profiles tool:

1. Select or open the component image (or images) in the Maestro work area.
2. Select **Line Profiles** from the **Tools** menu. The **Line Profiles** dialog box opens.
   - Ten rows in the color library let you draw up to ten profile lines on multiple monochrome images.
   - Each line profile appears as a plot in the Spectral Graph.
   - You can change the Spectral Graph Scale to either “Normalized” or “Scale To Max.”
   - The **Clear All** button is used to remove all profile lines from the image(s) and empty the Spectral Graph.
3. To draw a profile line, click the **Draw** button on the first row (Red) of the color library, and then use the mouse pointer to click and draw a line across the area of interest in the image. As you draw a line through bright portions of the image, the bright portions show up as peeks on the Spectral Graph.

4. Figure 50 shows three profile lines drawn on two component images of a mouse sample. (This is the same sample cube that was used during the “Practice Exercise” on page 45.)

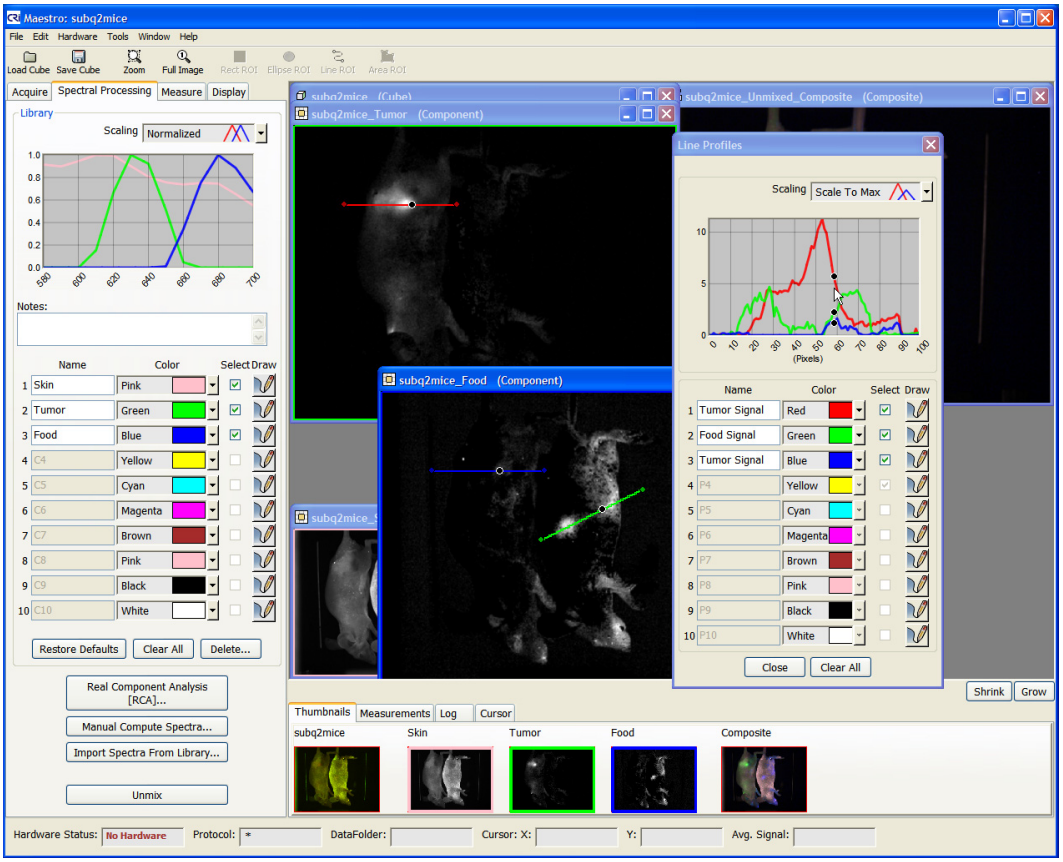


Figure 50. Line Profiles drawn on component images

Note the following attributes of the profile lines:

- The first profile line (Red) intersects the bright signal of the quantum dot-labeled tumor. Notice that the Red plot peeks for the pixels that are in the brightest area of the tumor.
- The second line (Green) intersects two less-bright signals caused by food and general autofluorescence in the control mouse.
- The third line (Blue) is a copy of the first line (Red), and is positioned at exactly the same location as the first line. (See “Working with Line Profiles” on page 76 for how to copy lines.) By comparing the Red and Blue lines, one can conclude that there is no tumor signal in the control mouse.
- When you move the mouse pointer across the Spectral Graph in the **Line Profiles** dialog box, you will notice a black dot appear on each line. These dots correspond with the dots on the profile lines on the images, and they can help you identify peeks and dips in the images’ spectral intensity.

The Line Profiles tool is for data exploration purposes only. The ability to export profile data has not yet been enabled.

Working with Line Profiles

After drawing line profiles on an image, you can copy, clone, move, and delete lines by right-clicking on them.

To copy line profiles to the clipboard:

1. Right-click on the image that contains the lines you want to copy, and then select **Copy Line Profiles To Clipboard** from the pop-up menu. This feature copies all profile lines in the image onto the clipboard. If you want to copy a single line to another image, simply drag and drop the line onto the other image. (Be sure to deselect the **Draw** tool before attempting to drag and drop the line.)

Note: When dragging a copy of a line to another image, the line will not move while you drag the mouse pointer. However, the copy will appear on the new image when you release the mouse button at the destination.

2. Right-click inside the image to which you want to paste the line profiles and select **Paste Line Profiles From Clipboard**.
3. All line profiles on the clipboard are pasted into the current image. Each new line profile is assigned the next row/color in the color library, and all lines are plotted in the Spectral Graph.

To clone a line profile:

1. Right-click on the line you want to clone and select **Clone Line Profile** from the pop-up menu. (Cloning creates an exact copy of the original that you can move to another area within the current image. You cannot move a clone to another image.)
2. The mouse pointer changes to a hand with a floating clone of the original line attached to it. Move the clone and click to release it at the desired location.

To move a line profile:

1. Right-click on the line you want to move and select **Move Line Profile** from the pop-up menu.
2. The mouse pointer changes to a hand with the floating line attached to it. Move the line and click to release it at the desired location.

To delete a line profile:

1. Right-click on the line you want to delete and select **Delete Line Profile** from the pop-up menu.
2. The line is removed from the image, the selection is removed from the color library, and its profile plot is removed from the Spectral Graph.

Chapter 7

Quantifying Results

The **Measure** panel is used to draw measurement regions and set measurement parameters. The **Measurements** page at the bottom of the Maestro work area displays the measurements of regions drawn on component images. This chapter explains each of the tools used for measuring regions on component images.

Topics in this chapter: **Page**

- Measuring Regions 77
- Ignoring Smaller Regions 82
- Adjusting Region Transparency and Color 82
- Understanding Region Measurements 83

Measuring Regions

Finding Regions Automatically Using Threshold Segmentation

1. Select the component (or any other single-plane) image to be measured and make sure the **Measure** panel is visible.
2. In the **Threshold Segmentation** box, click the **Auto Calculate Threshold** button. The Maestro software will attempt to find a threshold level that is appropriate for the image.

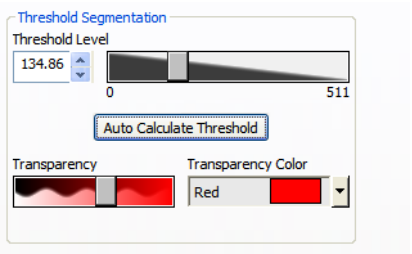


Figure 51. Threshold Segmentation

3. Pixels with intensity values below the specified threshold are ignored. If you are not satisfied with the auto calculated threshold level, you can enter a new value in the **Threshold Level** field by using the slider or manually typing in a value. You will see the region(s) change as you edit the threshold level.

4. After a threshold is set, the image will have an overlay showing the regions that were measured, as shown in Figure 51).

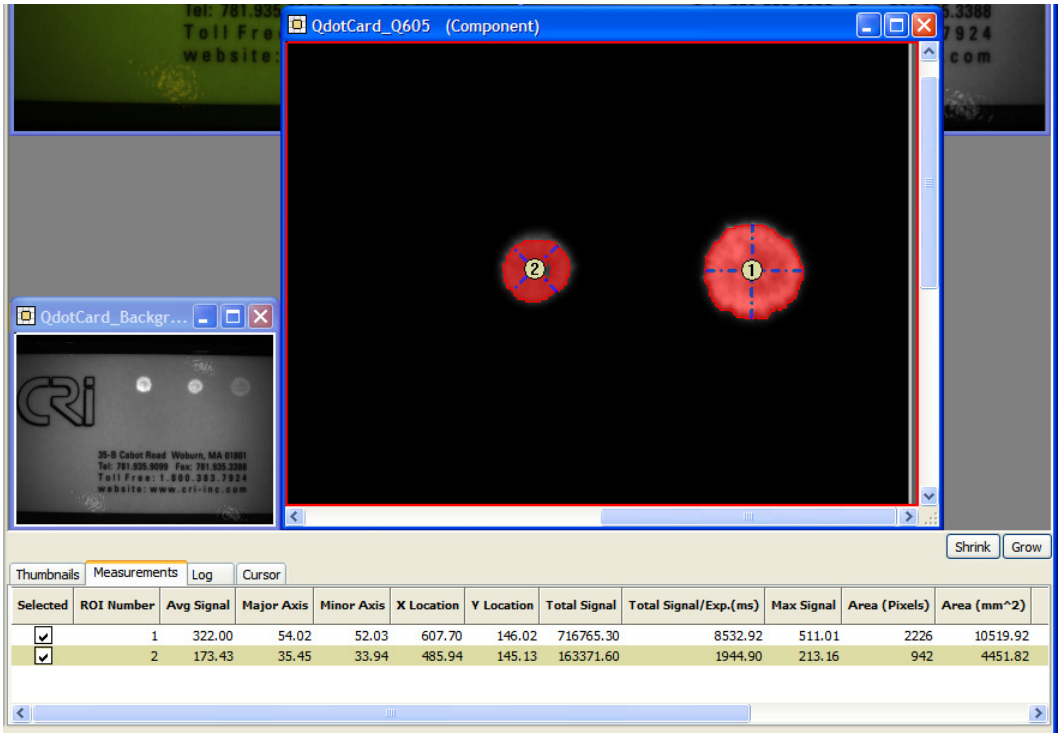


Figure 52. Measured Threshold Regions

The dashed lines drawn on top of each region show the region's major and minor axis drawn through the center of gravity.

5. If there are too many or too few measurement regions, you may need to adjust the minimum number of connected pixels (see “Ignoring Smaller Regions” on page 82).

6. You can adjust the region’s transparency or change its color (see “Adjusting Region Transparency and Color” on page 82).

Manually Drawing and Modifying Regions

The Maestro software provides tools for manually drawing and modifying measurement regions. These tools are located in the **Manual Draw Regions** box on the **Measure** panel:

To draw a region:

1. Click the **Draw** button to enter draw mode.
2. Select the draw tool shape by selecting from the toolbar or right-clicking the image and selecting from the pop-up menu (rectangle-, ellipse-, line-, or area-ROI).
3. Use the mouse pointer to draw new regions of the image to measure.
4. Each region is assigned an ROI number and is listed in the **Measurements** page at the bottom of the screen. Measurement regions are numbered and sorted according to their size, with the largest region first, and the smallest last.

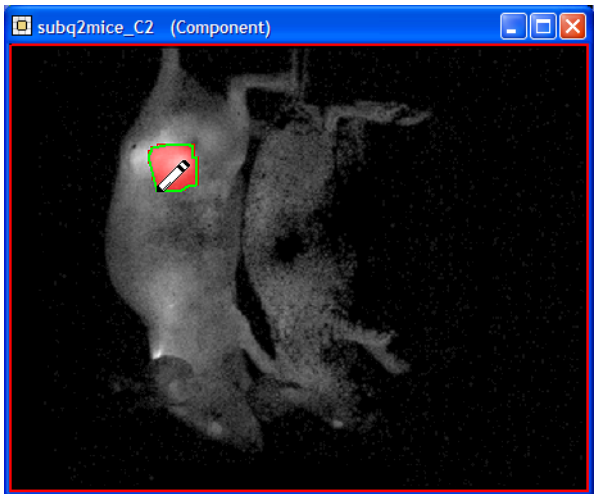


Figure 53. Manually Drawing a Region

To erase part of a region:

1. Click the **Erase** button to enter erase mode.
2. Select the erase tool shape by selecting from the toolbar or right-clicking the image and selecting from the pop-up menu (rectangle-, ellipse-, line-, or area-ROI).
3. Use the mouse pointer to remove pixels from existing regions.

To clear all regions:

Click the **Clear** button to remove all measurement regions.

To move or delete a region:

Right-click on the region you want to move or delete and select from the pop-up menu.

Cloning Regions

The Maestro software's ability to clone regions makes it easy to directly compare control regions and fluorophore regions of an image. Once you clone a region, you can then move the clone to another area of the image to compare the fluorescence signals.

To clone a region:

1. Right-click on the region you want to clone and select **Clone Region** from the pop-up menu. A clone of the region is created.
2. Move the clone to the desired area of the image. The clone will become the next sequentially numbered measurement region.

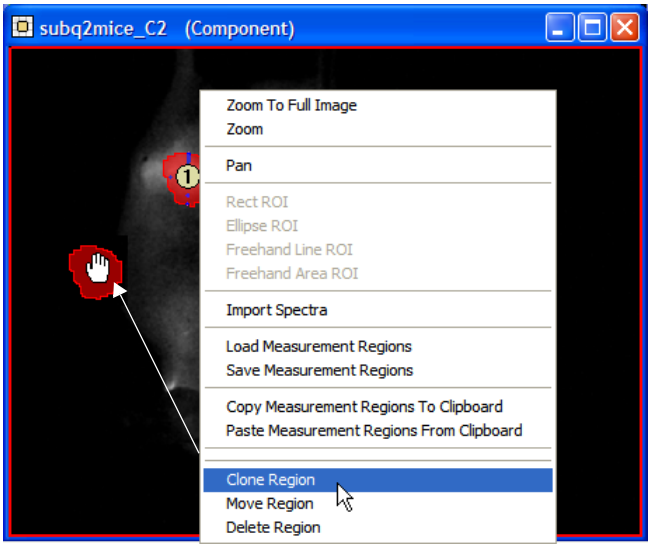


Figure 54. Cloning a Region

Dragging a Copy of a Region to Another Image

You can drag a copy of a region from one component image to another. When you drag a region to another image, the Maestro software automatically pastes the region at the exact same location in the new image. This feature is useful for comparing regions across two or more composite images.

To drag a region to another image:

1. Click on the region you want to copy, and while holding down the mouse button, drag it to the new image.
2. Release the mouse button anywhere in the new image. The copied region will automatically snap into position, corresponding to the position of the original.

Copying all Regions to the Clipboard

The Maestro software can copy all existing regions from an image to the clipboard. You can then paste the copied regions from the clipboard to other images. (All regions in the

image are copied with this feature. See “Dragging a Copy of a Region to Another Image” on page 80 if you want to copy a single region.)

To copy all regions to the clipboard:

1. Right-click on the image that contains the regions you want to copy, and then select **Copy Measurement Regions To Clipboard** from the pop-up menu.
2. Right-click inside the image to which you want to paste the regions and select **Paste Measurement Regions From Clipboard**.
3. All measurement regions on the clipboard are pasted into the current image. Each new region is assigned a ROI number, and its measurement data displays in a new row of the **Measurements** page at the bottom of the Maestro window.

Note: The ROI numbers can change from image to image depending on what other regions are present in each image.

Saving and Loading Regions

If you save your measurement regions, you can easily load them to other images. This is useful for comparing control regions and fluorophore regions across multiple images.

To save a region:

1. Right-click anywhere on the image (but not within a region) and select **Save Measurement Regions** from the pop-menu.
2. In the **Choose Measurement Regions** dialog box, enter a descriptive name, and click **Save** to save the measurement region(s).

To load a region to an image:

1. Right-click anywhere on the image (but not within a region) and select **Load Measurement Regions** from the pop-menu.
2. The region(s) will be loaded to their exact pixel location when they were saved. You can move the region(s) by right-clicking them individually and selecting **Move Region**.

Obtaining Accurate Measurements

The Maestro software calculates each region’s area in pixels and in mm^2 based on the current binning and stage height settings on the **Measure** panel. Therefore, these settings must be correct for the mm^2 measurement to be accurate.

If the current unmixed cube is a CRi format “Image Cube,” the Maestro software automatically displays (on the **Measure** panel) the binning used to acquire the cube. This setting cannot be changed. The stage height defaults to the stage height specified in the hardware settings prior to acquiring the cube. The Maestro software lets you edit this value in case the corresponding stage height was not entered correctly in the hardware settings.

TIFF cubes do not save binning and stage height specifications. If you load and unmix a TIFF cube, the binning value (on the **Measure** panel) defaults to 1x1 and the stage height defaults to the current stage height specified in the hardware settings. For TIFF cubes, you can change both of these values.

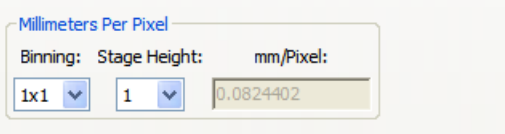


Figure 55. Millimeters Per Pixel

To change the binning or stage height

Select from the **Binning** or **Stage Height** drop down boxes. (Binning can be changed for TIFF cubes only.) The Maestro software calculates and displays the new mm/Pixel value automatically, based on the calibrated pixel size (see "Calibrating Pixel Size" on page 104).

Ignoring Smaller Regions

The **Measure Objects** box on the **Measure** panel is used to specify a minimum region size based on its number of pixels. Only regions that are larger than the **Min. Connected Pixels** value are considered. The Maestro software ignores regions that have fewer than this number of pixels.

Figure 56. Minimum Connected Pixels

Use this control to change the size of the regions to filter out. This setting does not effect manual draw regions.

Hiding Region Labels

Numbered region labels correspond to the ROI Numbers on the **Measurements** page at the bottom of the Maestro work area. Sometimes a region label obscures important data or makes it difficult to see the region clearly. You can have the Maestro software draw or hide numbered labels on regions within an image.

A check-mark in the **Draw Region Labels** check box (Figure 56) will show the region labels that are normally drawn on top of the region. Clearing the check-mark hides these labels.

Adjusting Region Transparency and Color

1. Use the **Transparency** slider (in Figure 51) on the **Measure** panel to change how much measurement regions block the image behind it. Moving this slider all the way to the

left makes the region transparent (except for its outline). Moving the slider all the way to the left fills the region with the selected color.

2. If you want to change the transparency's color, select a color from the **Transparency Color** drop down box.

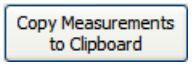
Understanding Region Measurements

Each region's measurements are displayed as rows on the **Measurements** data page near the bottom of the Maestro work area (Figure 52). The following information is displayed:

- The **Selected** column indicates which measurement regions are displayed on the image. Clear the check box from a measurement row to remove its region from the image.
- The **ROI Number** for each measurement region corresponds to the number displayed in the center of the region drawn on the image.
- **Average Signal** is the average value of the pixels in the region.
- **Major Axis** is the length of the minimum area bounding box enclosing the region.
- **Minor Axis** is the width of the minimum area bounding box enclosing the region.
- **X Location** is the center of gravity's x coordinate.
- **Y Location** is the center of gravity's y coordinate.
- **Total Signal** is the sum of all the pixel values in the region.
- **Total Signal/Exp.(ms)** is the Total Signal divided by exposure time in milliseconds.
- **Max Signal** is the maximum pixel value included in the region.
- **Area (Pixels)** is the number of pixels included in the region.
- **Area (mm^2)** is the size of the region in square millimeters. This measurement will only be displayed if the camera's pixel size has been calibrated (see "Calibrating Pixel Size" on page 104). Also, the accuracy of this measurement depends on setting the correct stage height and binning (see "Obtaining Accurate Measurements" on page 81).
- **Cube ID** is the ID entered in the **Sample ID** field on the **Acquire** panel when the cube was acquired.
- **Spectrum ID** is the ID of the spectrum used to unmix this image (entered from the **Spectral Processing** panel).
- **Cube Time Stamp** represents the time the cube was created.

Copying Measurement Data to the Clipboard

1. Make sure the measurement regions you want to copy are selected on the **Measurements** data page.
2. Click the **Copy Measurements to Clipboard** button in the **Measure** panel.



3. You can now switch to a program such as Microsoft Excel and paste the measurements into a worksheet.

Saving Measurement Data as a Text File

1. Make sure the measurement regions you want to save are selected on the **Measurements** data page.
2. Select **Save Measurements** from the **File** menu. The **Choose Measurement File** dialog box opens.
3. Select a location and enter a name for the text file. Click the **Save** button to save the new text file.

Appending Measurement Data

You can append new measurement data to end of an existing measurement data text file.

1. Make sure the measurement regions you want to append to a saved text file are selected on the **Measurements** data page.
2. Select **Append Measurements** from the **File** menu. The **Choose Measurement File** dialog box opens.
3. Select the file to which you want to append the measurement data and click the **Save** button.

Chapter 8

Customizing Spectral Displays

This chapter explains how to use the **Display** panel to change display parameters to obtain a clear view of an image.

Topics in this chapter **Page**

- Adjusting Brightness and Contrast Levels 85
- Saturation Mask 86
- Changing How a Cube is Displayed 87
- Changing How a Composite is Displayed 88

Adjusting Brightness and Contrast Levels

The controls in the **Display Mapping** box at the top of the **Display** panel are used to change brightness and contrast levels within images.

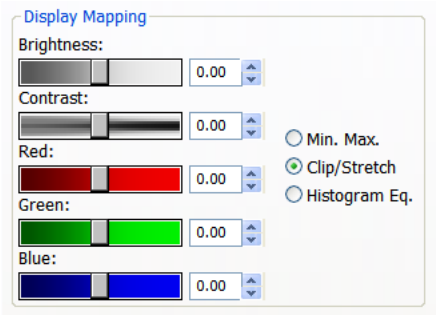
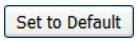


Figure 57. Display Mapping

- Four sliders allow you to adjust the following:
  - Brightness
  - Contrast
  - Brightness for the individual red, green, and blue color channels. These sliders are enabled only when a cube or composite image is selected.
- The **Min. Max.** radio button maps the minimum value in the image to 0, the maximum value to 255, and linearly interpolates in between those values. This stretches dark signals so they become visible.
- The **Clip/Stretch** radio button maps the lowest 0.01% of the pixels to 0, the highest 0.01% to 255, and linearly interpolates in between those values. This prevents a few bright or saturated pixels from skewing the display.
- The **Histogram Eq.** radio button maps the pixels so the histogram of the pixels have approximately the same number of pixels in each bin. This gives the best display of the whole dynamic range of dim and bright signals.

Returning to Display Defaults

If you want to return the display mappings to the default values, click the **Set to Default** button at the bottom of the **Display** panel.



Saturation Mask

Saturated pixels in your images reduce the accuracy of your unmixing and quantitation results. The Maestro software considers a pixel in a cube "saturated" if the pixel is saturated at any of the cube's wavelengths. Pixels in a component image remain saturated if they were saturated in the cube used to create the component. In a composite image, a pixel will be saturated if it was saturated in the component image.

If saturation is present in your image, use the **Saturation Mask** controls to reduce or eliminate the effects of the saturated pixels. These controls are found at the bottom of the **Display** panel.

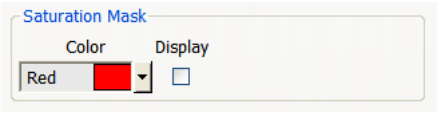


Figure 58. Saturation Mask

To apply a saturation mask:

1. Select the image to which you want to apply the saturation mask.
2. Select the color that matches the saturated pixel color.
3. Click the **Display** check box to apply the saturation mask to the image.

Changing How a Cube is Displayed

Computers display color through varying intensities of red, green, and blue. In order for the Maestro software to display an image cube, which has many more color planes, the Maestro software maps all colors in the cube to red, green, and blue planes.

When an image cube is active, the **Wavelength to RGB Map** box becomes visible in the **Display** panel. Wavelength ranges in the image cube are mapped to red, green, or blue to create the display image.

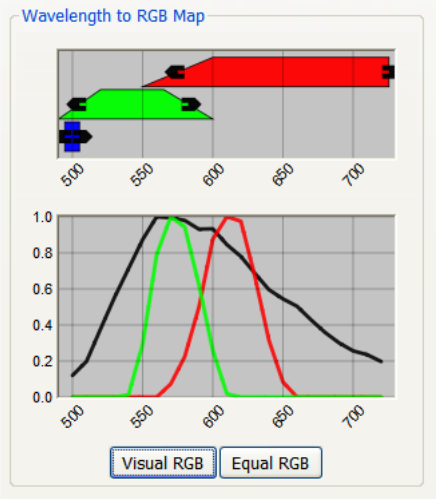


Figure 59. Wavelength to RGB Map

The plot below the bars shows the current spectral library. It will also show the spectrum underneath the cursor when it is moved over the display image.

The upper control has three bars, colored red, green, and blue. The x-axis represents the wavelength range of the cube. The wavelengths in the red bar are mapped to the red plane in the display image (green to green, and blue to blue).

You can move these bars by clicking in their interior and dragged to a new position. The bars can be resized by dragging the handles on the ends of the bars.

To View Visual RGB

Click the **Visual RGB** button to set the bars so they approximate the human eye.

To View Equal RGB

Click the **Equal RGB** button to set the bars so they divide the cube's wavelength range equally among the red, green, and blue planes.

Changing How a Composite is Displayed

The Maestro software displays a composite image by combining the displays—not the raw data—of the component images. When a composite image is active, the **Component Selection** box becomes visible in the **Display** panel.

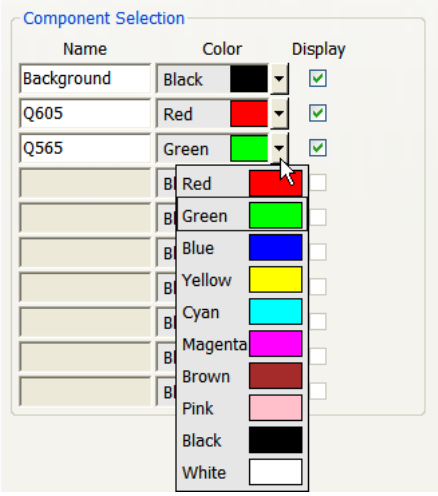


Figure 60. Component Selector

There is a row for each component image. Use the **Display** column to control which components are used to create the composite image. If you want to change the color used to display any of the component images, select a color from the component's **Color** drop down box.

This feature is very useful when you want to make the fluorophore signal of interest more clearly visible in a composite image. For example, you could exclude the Background component (thereby removing the autofluorescence) from the composite image. Doing this in combination with adjusting the **Brightness** and **Contrast** of the unmixed components (using the sliders in the **Display Mapping** box), can result in a clear bright image of the tumor, fluorophore, etc.

Chapter 9

Frequently Asked Questions

This chapter discusses FAQs and troubleshooting issues for the Maestro *in-vivo* imaging system.

Can I use the multispectral camera in my Maestro Imaging Module on my microscope?

Maestro *in-vivo* imaging systems are designed specifically for macro-imaging. Contact CRi to find out about our Nuance™ Multispectral Imaging Systems for microscopy.

What is the CCD sensor active area dimensions and the individual pixel size?

The active light sensitive area of the CCD is 1392 pixels in the horizontal direction and 1040 pixels in the vertical direction. Each pixel is 6.45 µm square.

Can I use my own lenses on a Maestro *in-vivo* imaging system?

The lens is built into the Maestro *in-vivo* imaging system. Other lenses cannot be used.

Why do I need an emission filter with the Maestro *in-vivo* imaging system?

Without an emission filter, specifically a longpass filter, excitation light will bleed through the Maestro optical system and cause artifacts. See “Chapter 1, Introduction to Multispectral Imaging Theory and Concepts.”

My specimens are sometimes unevenly illuminated by the lighting system. What can I do about this?

It is possible to accidentally set incompatible heights that can result in uneven or suboptimal illumination. Make sure the proper height settings for the specimen stage

and fiber-optic illuminators are used. Also, be sure to use Flat Fielding when acquiring cubes (see “Flat Fielding” on page 58).

Why are my exposure times so long?

It is possible that there is too little light and the Maestro *in-vivo* imaging system needs to use a longer exposure time.

Make sure the fiber-optic illuminators are positioned properly and that the illumination module is properly set up and connected. Check that the illumination light source has not exceeded its useful life span. The light source should be replaced after 500 to 1000 hours of use.

Why are my exposure times so short?

The liquid crystal (LC) tuning element may have been moved out of the light path.

To correct this, make sure the thumb slider switch is in the far right position. The thumb slider switch protrudes from the ceiling inside the imaging module, at the top of the case near the base of the lens assembly.

When I switch on the near-infrared (NIR) viewing lights inside the imaging module, I don’t see anything appear to get brighter with my eyes. How can I be sure that the NIR illumination system is working?

Our eyes do not see NIR-wavelength light very well, so it is easy to mistakenly think the NIR interior lights are not working, when in fact they are.

Review the section, “Interior Lights” on page 17 for a detailed explanation of the white and IR LEDs and how to verify they are working.

What fluorescence filters sets do I need?

Refer to “Appendix C, Filter Selection Guide,” or contact your local authorized distributor or CRi to discuss your needs. The filters used in the Maestro *in-vivo* imaging system are designed as matched sets for optimum performance.

I installed the Maestro software, but when I launch the application, it doesn’t recognize the hardware.

If the imaging module was left on and connected to the computer during installation of the software, Microsoft Windows may not have been able to install the correct drivers for the camera.

Disconnect the imaging module from the computer. Make sure the installation CD is in the CD drive, then reconnect the imaging module to the computer. Windows should detect the new hardware and begin the Hardware Installation Wizard. Follow the prompts to install the drivers. See “Installing Maestro Software” on page 101.

Appendix A. System Specifications & Dimensions

Operating Specifications

Note that these specifications represent typical Maestro *in-vivo* imaging systems as of August 2006 and are subject to change.

Parameter	Maestro VIS	Maestro GNIR
Wavelength range	420 to 720 nm	500 to 950 nm
Bandwidth, FWHM	Optimized for *in-vivo* fluorescence	
Image sensor format	2/3-inch CCD	
Image sensor pixel-count	1.3 mega pixels	
Cooling	<5 °C above ambient	
Sample area	1-1/3 in x 1 in. to 4 in. x 3 in.	
Typical acquisition time[1]	15-frame image cube in 5 to 10 seconds	
Electrical supply	100 to 240VAC, 800W, 50/60Hz	
Operating environment	Indoor	
Operating temperature	15 °C to 40 °C	
Operating humidity	65%, non-condensing	
Operating Altitude (maximum)	2000 meters	
Storage temperature	-10 °C to 55 °C	
Storage humidity	100%, non-condensing	
Pollution degree	2	

[1] Acquisition time depends on a number of factors, including bandwidth of the Maestro *in-vivo* imaging system and the amount of light emitted, reflecting off, or transmitted through the specimen at each wavelength of interest. The Maestro *in-vivo* imaging system is designed to be able to "dwell" at individual wavelengths if it determines that more light is needed to produce images with adequate spatial and spectral detail. The system is **not limited** by a need to acquire images using identical exposure times at every wavelength.

Parameter		Maestro VIS	Maestro GNIR
Filter sets[1]		See Filter Selection Guide	
Lamp cartridge lifetime (estimated hours)		~500 hrs (lamp will then give approximately 50% of original intensity)	
Light delivery system		Fiber-optic	
Input/output port diameter[2]	Set 1	1.0 in.	
	Set 2	0.5 in.	
	Set 3	0.25 in.	
Imaging module dimensions		18.6 in. wide x 16.3 in. deep x 37.4 in. high	
Weight[3]	Imaging module	100 lbs. (45 kg)	
	Illumination module	20 lbs. (9 kg)	

[1] Filter sets must be purchased separately. Contact CRi or your authorized local distributor for pricing and availability.

[2] For anesthesia delivery system (not supplied). Unused ports may be kept covered with the supplied plugs.

[3] Does not include workstation computer.

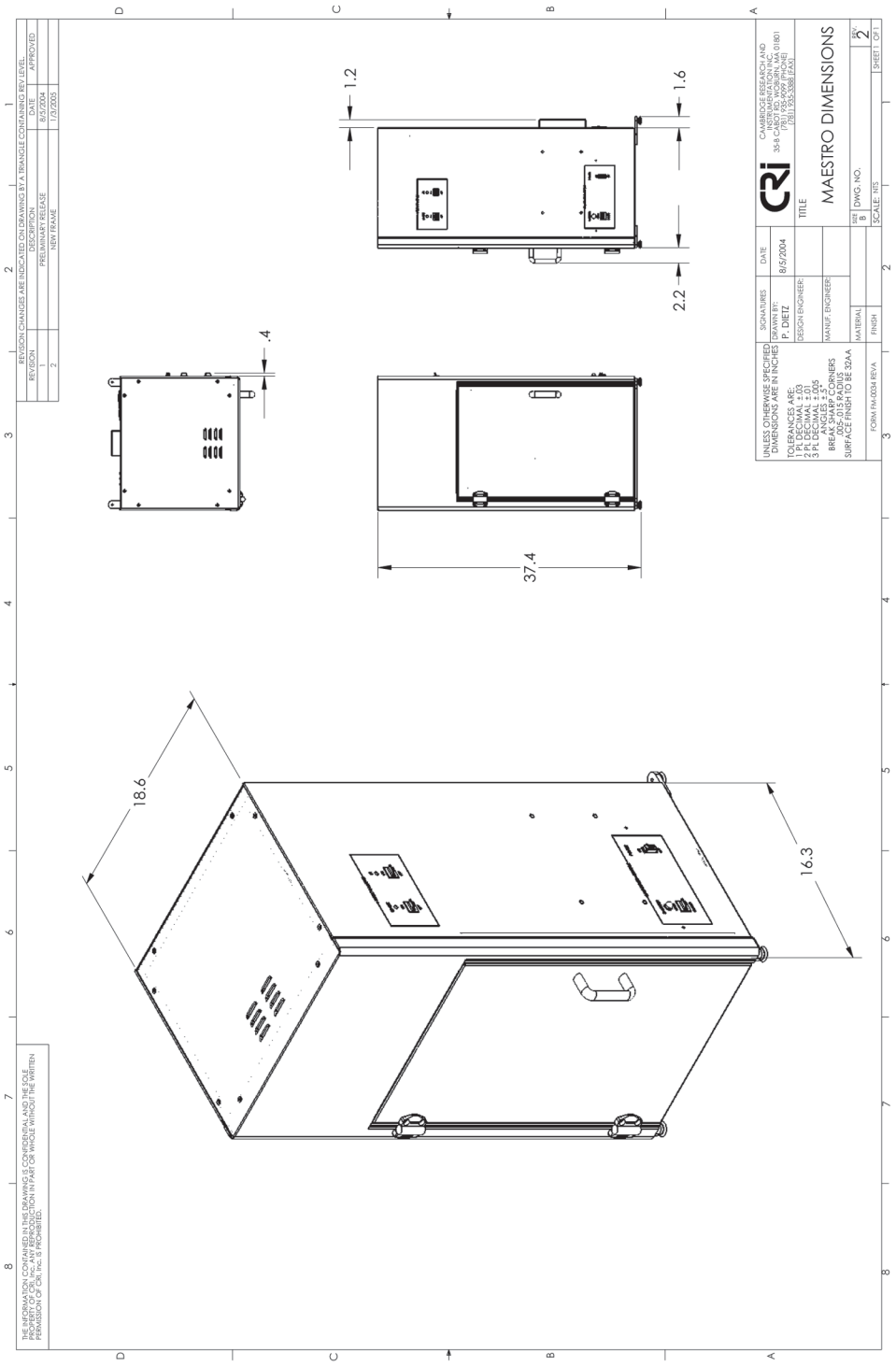
Computer System Requirements

Processor	3.0 GHz or higher Intel™ Pentium™ 4 class or Intel Centrino™
RAM (minimum)	2 GB
Hard disk drive (minimum)	160 GB
CD-ROM	writable CD-ROM drive
Operating system	Microsoft® Windows® XP Professional or Windows 2000 Professional (English Language Version)
Display (minimum)	24-bit color, 1280 by 1024 pixels
Computer Interface	One available USB 2.0 interface

Mechanical Dimensions

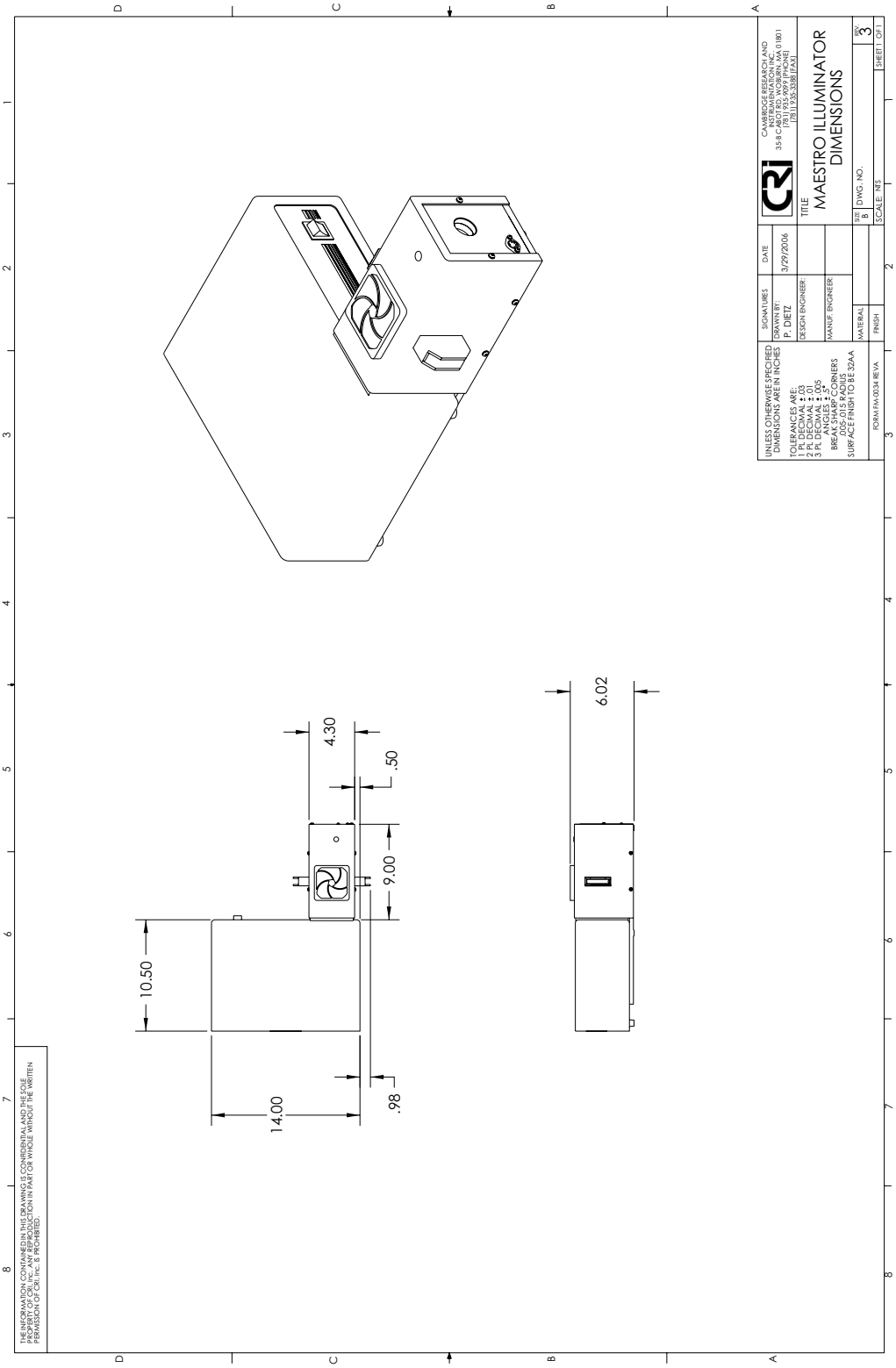
Imaging Module

This diagram provides external dimensions of the imaging module in inches. Dimensions are subject to change without notice.



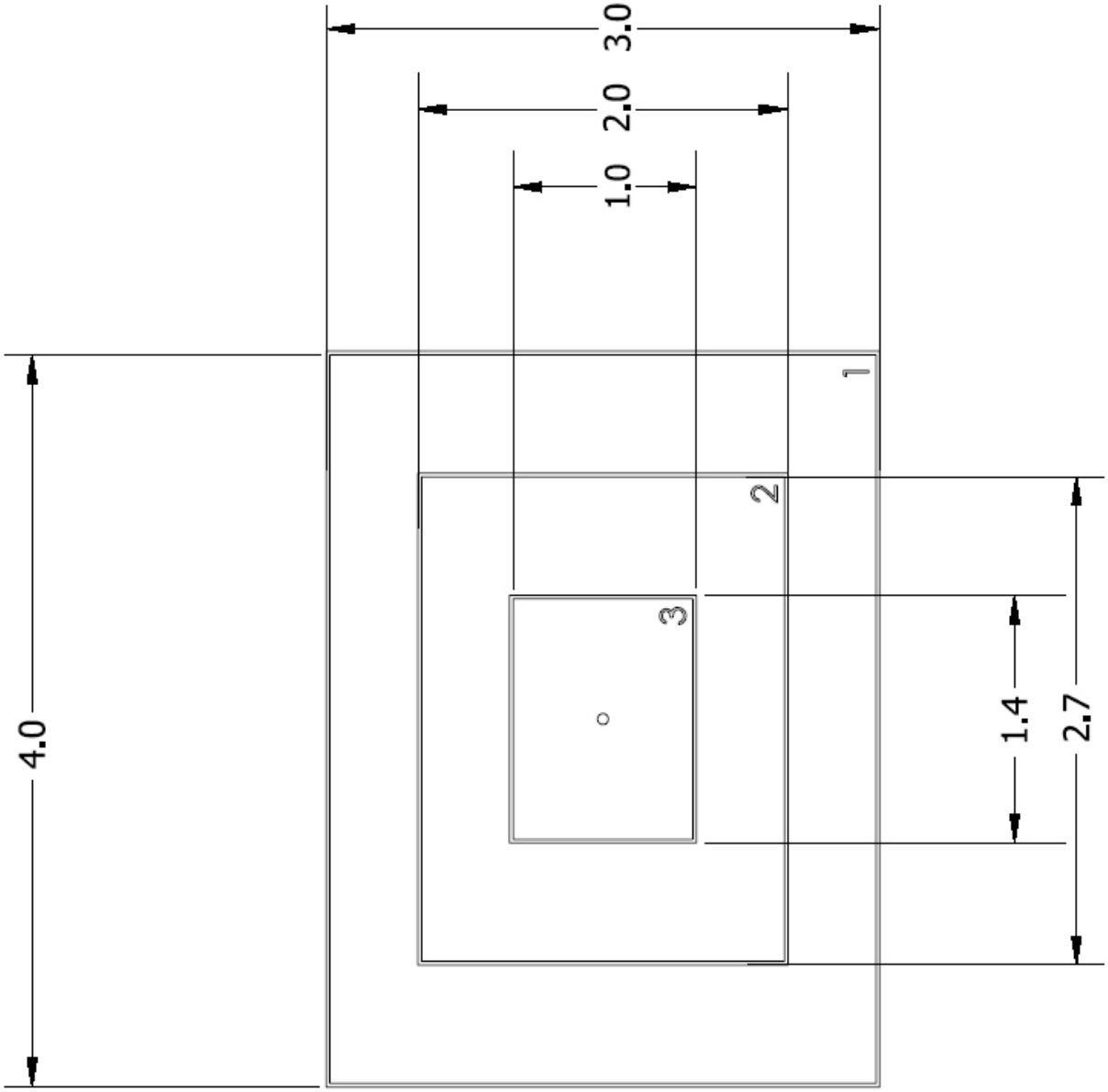
Illumination Module

This diagram provides external dimensions of the illumination module in inches. Dimensions are subject to change without notice.



Specimen Stage

This diagram provides a 1:1 scale drawing of the field of view outlines on the specimen stage. It is intended to assist you in determining if a particular specimen meets the size requirements of the system.



Appendix B. System Setup & Installation

Maestro *in-vivo* imaging systems consist of an Imaging Module and an Illumination Module, configured at the factory for either 420 to 720 nm or 500 to 950 nm. This chapter describes how to set up both modules, along with the Workstation Computer and LCD monitor for superior fluorescence *in-vivo* imaging. Each component requires interconnecting cables and electrical power.

Unpacking the Maestro *In-Vivo* Imaging System

The Maestro *in-vivo* imaging system is transported in a wooden shipping crate that is built to protect the system during shipping. Additional boxes containing the computer and monitor (if purchased) are also placed on the pallet. The entire pallet and its contents are wrapped with plastic material to facilitate shipping the entire system as one unit.

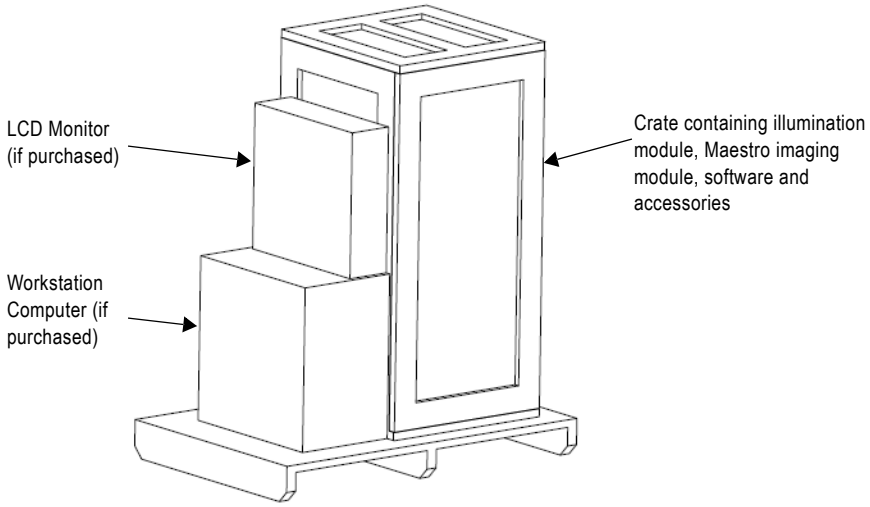


Figure 61. Maestro Shipping Crate and Pallet

1. When you are ready to install the system, remove the plastic wrapping material, then remove the monitor and computer boxes (if purchased) from the pallet.

   The shipping crate should be opened by removing the marked screws on the front side and top of the crate.

Note: CRi recommends that you store the pallet and all parts of the crate, including the screws, in case the system ever needs to be shipped again. To protect against possible damage during transport, the system should only be shipped in its original packaging.

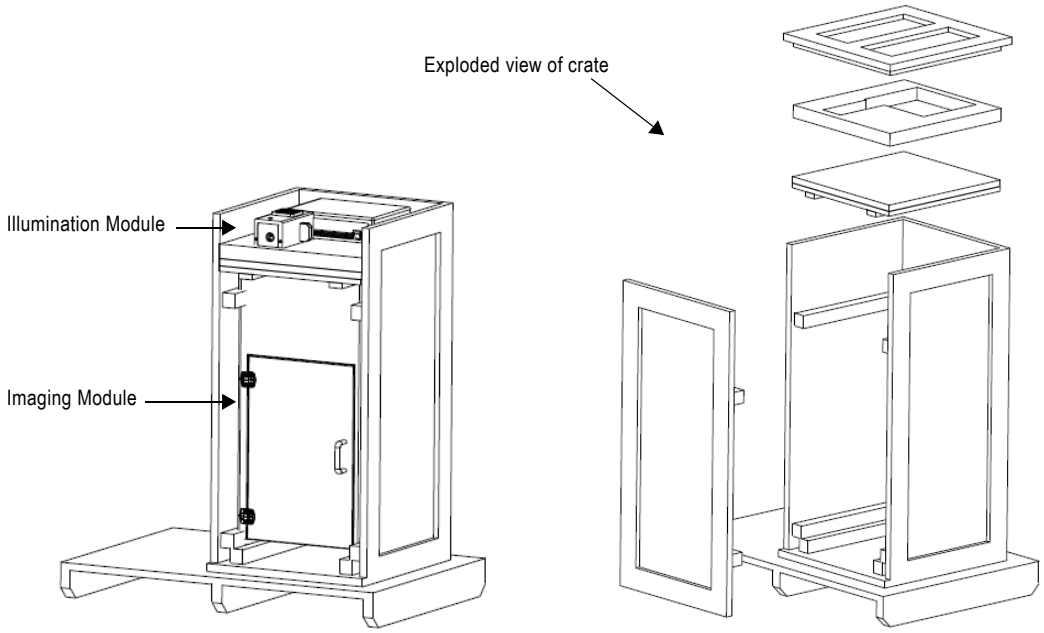


Figure 62. Removal of Maestro and components from shipping crate

2. With the front and top of the shipping crate removed, identify and remove the packages containing the cables, user manual, installation software, specimen pad, and the CRi business card with QDot spots.
3. Carefully lift the illumination module from the top of the Maestro cabinet, and set it aside. Remove the plywood panel and the foam blocking that supports it.
4. The Maestro imaging module can now be removed. At least two people should remove the imaging module by carefully sliding it out of the crate.

Note: The imaging module is shipped in a plastic bag, which may cause it to feel slippery. **Be careful not to drop the unit** as it clears the crate supports.

5. Remove the plastic bag and remove the shipping tape from the door. Place the imaging module on the table or bench where it will be installed.
6. Open the imaging module, and carefully remove the foam blocks that are contained within the chamber. These blocks are used during shipping to protect the illumination and stage components from shipping damage.

Setting Up the Maestro *In-Vivo* Imaging System

Figure 63 illustrates a typical setup configuration.

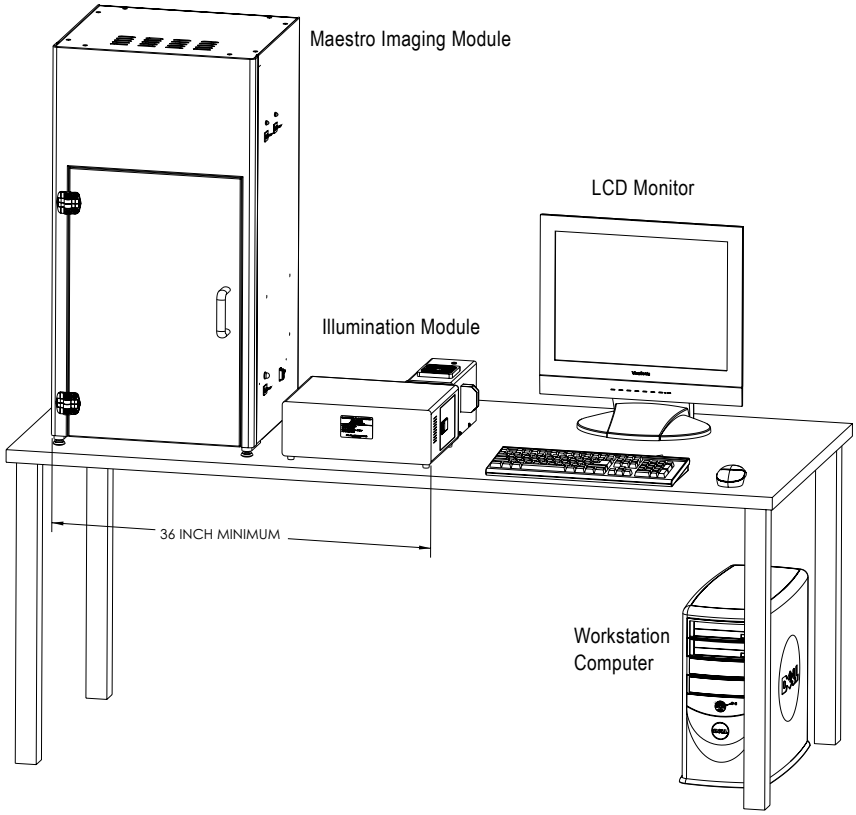


Figure 63. Basic Maestro Site Requirements & Configuration

1. Position the imaging module on the table or bench as shown in Figure 63.
2. Position the illumination module on the left side of the imaging module so its rectangular projection is positioned toward the back of the imaging module. Allow at least 36 inches of counter space from the left side of the illumination module to the right side of the imaging module.
3. Connect the fiber-optic light guide from to the illumination module. The fiber-optic guide is permanently attached to the imaging module and receives light from the illumination module.
    a. On the top surface of the rectangular projection of the illumination module is a small white plastic plug that covers the fiber-optic clamp screw (see Figure 64). Pry the plug up with your fingernail or other tool that will not scratch the paint to expose the locking screw.
    b. Use a phillips head screwdriver to loosen the screw several turns.
    c. Remove the fiber-optic guide from its plastic cable clamps on the back of the imaging module. Remove the protective cap from the end of the fiber-optic guide.

d. Insert the fiber-optic guide into the guide port in the end of the rectangular projection (see Figure 64). Push the guide in until it stops. The metal end of the guide should seat completely within the housing.

e. Tighten the clamp screw and replace the plastic plug.

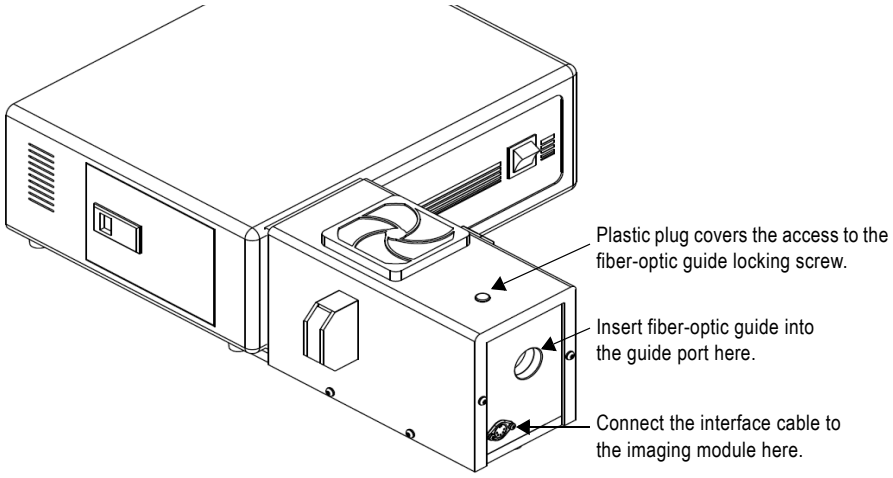

Plastic plug covers the access to the fiber-optic guide locking screw.
Insert fiber-optic guide into the guide port here.
Connect the interface cable to the imaging module here.

Figure 64. Maestro Illumination Module

4. Use the supplied interface cable to connect the illumination module to the imaging module: Plug the cable into its port on the illumination module (see Figure 64) and the ILLUMINATOR CONTROL port on the back of the imaging module.

5. Use the supplied USB cable to connect the imaging module to the workstation computer: Plug the cable into the square USB 2.0 port on the back of the imaging module (see Figure 65) and any high-speed USB 2.0 port on the computer.

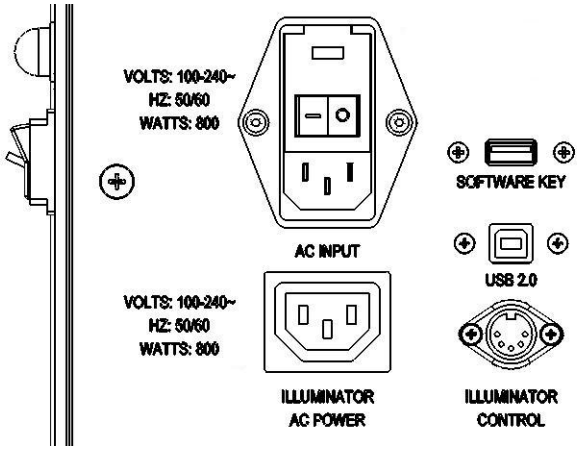


Figure 65. Maestro Imaging Module, Back Panel Connections

Note: The USB port labeled “Software Key” is no longer used to connect a software key. This port may be used as a general purpose USB port.

6. Setup the LCD monitor, keyboard, and mouse on the table with the Maestro *in-vivo* imaging system and connect them to the workstation computer. Detailed instructions for these connections are available in the materials supplied with the computer.

Caution! The imaging module, workstation computer, and monitor require connection to a surge protected electrical supply. Before connecting system components to the electrical supply, verify that all power switches are in their OFF positions.

7. Verify that the electrical power supply is the correct rating (see "Appendix A, System Specifications & Dimensions"), then plug each component's power cord into the electrical supply.

Installing Maestro Software

Important! If you purchased a computer from CRi to use with your Maestro *in-vivo* imaging system, the Maestro software is pre-installed on the computer by CRi. There is no need to perform a software installation unless you are installing the software on your own computer, or if the contents of your computer's hard drive have been erased, or if the system software has been "rolled back" using Microsoft Windows system administration tools.

Important! Before following this procedure, CRi highly recommends that you familiarize yourself with the basic operation of the Microsoft Windows operating system (e.g. saving and deleting files and folders, startup and shutdown procedures, and navigating through the contents of your hard disk drive).

If you need to install or re-install the Maestro software, follow the procedure below:

1. Verify that your computer's hardware is in good working order. If using a computer not supplied by CRi, make sure that CRi's minimum specifications have been satisfied. (Refer to "Operating Specifications" on page 91.) If you are not sure, call your local authorized distributor or CRi directly.
2. Verify that your computer's operating system is also in good working order. CRi recommends maintaining an up-to-date operating system, using procedures recommended by Microsoft and the computer manufacturer.
3. If the Maestro imaging module has never been connected to the computer, it is best to wait until after the software installation is finished before connecting the module. Also, make sure the imaging module is switched off.
4. If the Maestro imaging module is connected to the computer, disconnect it now and switch its power off. **Do not skip this step:** After the software is installed, Windows needs to detect the Maestro hardware to install the correct drivers. In order for this to

occur, you need to connect the imaging module to the computer after the software installation is complete.

5. Exit all other Windows programs that may be running.
6. Insert the Maestro software installation CD into the computer's CD drive and proceed with one of the installation options listed below:
   a. To install the Maestro software from the CD, follow the onscreen instructions if the installation wizard starts automatically. If the installation wizard does not start automatically, access the CD drive and double-click the icon labeled **Setup.exe** to start the installation. Follow the onscreen instructions to install the software.
   b. If you received a software update from CRi by Email or by downloading it from CRi's FTP site, and you do not intend to install from the CD, cancel the installation wizard, but leave the CD in the drive. Windows will need to copy drivers from it during the update. Double-click the installation icon and follow the onscreen instructions to install the downloaded software.
7. During the installation, you will be prompted to select the hardware configuration you own. Select the "Maestro *in-vivo* imaging system" option.

Note: If you are upgrading from an earlier version of the Maestro software for a system with a USB 2.0 interface, your current settings will be backed up.

8. When the software installation is finished, switch on the imaging module and plug its USB cable into the computer's USB port.
9. Windows will automatically detect the new hardware. The **New Hardware Wizard** will ask if you want Windows to connect to Windows Update to search for software.
10. Select "No, not at this time" and click **Next**.
11. Make sure the installation CD is in the CD drive, select the "Install the software automatically (recommended)" option, and click **Next** to continue.
12. A **Hardware Installation** message will alert you that the "CRi unconfigured device has not passed Windows logo testing to verify its compatibility with Windows XP." You should ignore this message by clicking the **Continue Anyway** button.
13. The **New Hardware Wizard** will install the drivers from the installation CD.
14. When the installation is finished, the wizard prompts you to restart the computer.
15. Restart the computer. The hardware will not work until you restart the computer.
16. Software and Hardware installation is now complete.
17. See "Launching the Maestro Application" on page 22 for how to launch and activate the Maestro software.

Activating Maestro Software

The first time the Maestro software is launched, an activation code must be entered to activate the software. Connect the computer to the Internet if an internet connection is available. (Activation is quickest if the computer has an active Internet connection.) Then select one of the following options in the **InstallShield Wizard** activation dialog box.

Note: Keep in mind that the serial number (i.e., activation code) that came with your Maestro software allows you to install and activate the software on a limited number of computers. The actual number of computers depends on your purchase agreement with CRi.

To activate Maestro software via the Internet:

1. In the **InstallShield Wizard** activation dialog box, select "I have a serial number and I want to activate Maestro" and click the **Next** button.
2. Enter the serial number found on the Maestro software CD case and click **Next** to activate.

   Important: This is the serial on the software CD. **Do not** enter the serial number found on the back of the Maestro imaging module.
3. If the computer has an Internet connection, your Maestro software will be activated within a few seconds.

To activate Maestro software via Email:

If there is no Internet connection, you can still activate the Maestro software by Email.

1. In the **InstallShield Wizard** activation dialog box, select "I have a serial number and I want to activate Maestro" and click the **Next** button.
2. Enter the serial number found on the Maestro software CD case and click **Next** to activate.
3. You will receive an error message stating that the Activation Request Code could not be sent. Click **OK**.
4. When the **Serial Number** dialog box reappears, make sure the serial number is correct and select the "Activate by Email" option. Click **Next**.
5. A dialog box containing an Email link prompts you to "Email an Activation Request Code to CRi". Use any computer to Email the code to the address indicated. When you receive the Activation Response Code, enter it in the activation dialog box and click **Next** to activate the software.

Calibrating Pixel Size

To take measurements using mm^2 units, the Maestro software needs to know the camera's pixel size. This procedure requires that you have already acquired a clear and focused monochrome image of a metric scale or ruler at least 100 mm long, with the stage height on setting #1, and the CCD binning set to 1x1.

1. With the monochrome image of the metric scale or ruler loaded, select **Calibrate Pixel Size** from the **Hardware** menu. The **Distance Calibration** dialog box opens.

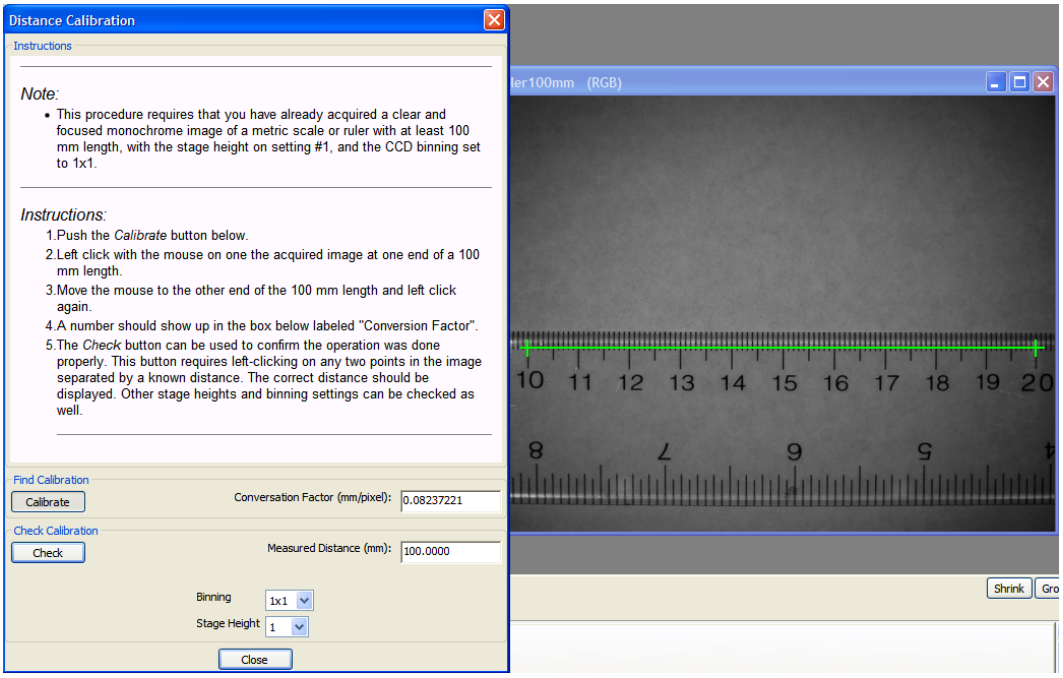

Distance Calibration
Instructions
Note:
This procedure requires that you have already acquired a clear and focused monochrome image of a metric scale or ruler with at least 100 mm length, with the stage height on setting #1, and the CCD binning set to 1x1.
Instructions:
1.Push the Calibrate button below.
2.Left click with the mouse on one the acquired image at one end of a 100 mm length.
3.Move the mouse to the other end of the 100 mm length and left click again.
4.A number should show up in the box below labeled "Conversion Factor".
5.The Check button can be used to confirm the operation was done properly. This button requires left-clicking on any two points in the image separated by a known distance. The correct distance should be displayed. Other stage heights and binning settings can be checked as well.
Find Calibration
Calibrate
Conversation Factor (mm/pixel):
0.08237221
Check Calibration
Check
Measured Distance (mm):
100.0000
Binning
1x1
Stage Height
1
Close
Shrink

Figure 66. Distance Calibration

2. Position this dialog box on the screen so the image of the ruler is visible.
3. Select 1x1 in the **Binning** drop down box, and select 1 in the **Stage Height** drop down box.
4. Click the **Calibrate** button.
5. Click with the mouse on the acquired image at one end of a 100 mm length.
6. Move the mouse to the other end of the 100 mm length and click again.
7. The number of millimeters per pixel displays in the **Conversion Factor** field.
8. You can use the **Check** button to confirm that the calibration is accurate. To use the Check function, obtain an image with points separated by known distances. Then click the **Check** button and click on any two points in the image separated by a known distance. The correct **Measured Distance** should be displayed. Other stage heights and binning settings can be checked as well using this function.

Tests for Ensuring Proper Operation

The best test to make sure everything is working properly is to take images of the CRi business card included with each Maestro *in-vivo* imaging system. The quantum dots on its front should show up clearly, and you can test the Maestro software's unmixing feature. See "Chapter 5, Acquiring Spectral Images" for detailed instructions.

Using Third-Party Computers with the Maestro Software

CRi supplies a computer with each Maestro *in-vivo* imaging system. If you intend to use a third-party computer system for offline data processing, please consider the following parameters for optimum performance:

- The faster the computer CPU, the faster certain operations in software will be accomplished. See "Appendix A, System Specifications & Dimensions."
- More hard drive space allows more and larger datasets to be acquired and stored.
- At least two GB of RAM is recommended for data processing.
- A USB 2.0 port is required.
- The US English versions of Windows® XP Professional or Windows 2000 Professional are the only supported operating systems.

Appendix C. Filter Selection Guide

Select which filters are optimal for your application. This table is current as of August 2006. Contact CRi or your authorized distributor for pricing and ordering information. The CRi Maestro *in-vivo* imaging system has many applications involving a variety of materials, such as probes, cell lines, animal specimens, etc. Certain of these applications and/or materials may require licensing under patents held by third parties. See "Notice to Purchaser" on page 113 for more details.

Fluorophore			Filter Set					
Name	Excitation (nm)	Emission (nm)	Blue	Green	Yellow	Red	Deep Red	NIR
525 nm QDot®	< 500	525	X					
545 nm QDot®	< 520	545	X					
565 nm QDot®	< 540	565	X					
605 nm QDot®	< 580	605		X				
655 nm QDot®	< 620	655			X			
705 nm QDot®	< 650	705				X		
800 nm QDot®	< 750	800					X	
Alexa Fluor® 488	492	520	X					
Alexa Fluor® 500	500	530	X					
Alexa Fluor® 514	514	545	X					
Alexa Fluor® 532	532	554	X					
Alexa Fluor® 546	556	573	X					
Alexa Fluor® 555	555	580	X					
Alexa Fluor® 568	577	603		X				
Alexa Fluor® 594	590	618		X				
Alexa Fluor® 610	612	628		X				
Alexa Fluor® 633	632	650		X				
Alexa Fluor® 647	647	666		X				
Alexa Fluor® 660	668	698			X			
Alexa Fluor® 680	679	702			X			
Alexa Fluor® 700	700	719				X		
Alexa Fluor® 750	750	779					X	
Cy2™	498	506	X					
Cy3™	554	568	X					
Cy5™	649	666			X			
Cy5.5™	675	695			X			
Cy7™	743	805					X	
GFP	475	509	X					
FITC	490	525	X					
eGFP	498	515	X					
YFP	520	532	X					
Rhodamine	550	573	X					
RFP (dsRed)	558	583		X				
ICG	800	850						X

Color denotes usable filter set, **X** denotes recommended filter set

Maestro Filter Set	Part #	Excitation Filter	Emission Filter	Acquisition Settings*
Blue	M-MSI-FLTR-BLUE	445 to 490 nm	515 nm longpass	500 to 720 in 10 nm steps
Green	M-MSI-FLTR-GREEN	503 to 555 nm	580 nm longpass	550 to 800 in 10 nm steps
Yellow	M-MSI-FLTR-YELLOW	575 to 605 nm	645 nm longpass	630 to 850 in 10 nm steps
Red	M-MSI-FLTR-RED	615 to 665 nm	700 nm longpass	680 to 950 in 10 nm steps
Deep Red	M-MSI-FLTR-DEEP-RED	671 to 705 nm	750 nm longpass	730 to 950 in 10 nm steps
NIR	M-MSI-FLTR-NIR	710 to 760 nm	800 nm longpass	780 to 950 in 10 nm steps

* The ending wavelength can always be made longer if there is fluorescence beyond the suggested end point.

Removing & Installing Filters in the Filter Slider

Installing Emission Filters into an Emission Filter Slider

Emission filters must be installed in the filter slider with the correct top-to-bottom orientation. A small arrow on the side of each filter must be pointing downwards (towards the specimen stage when installed in the imaging module). After installing the filter, slide the keeper back into place and tighten the screw.

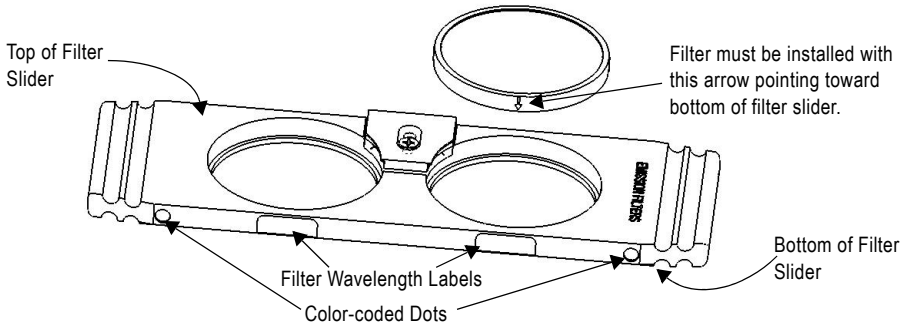

Top of Filter Slider
Filter must be installed with this arrow pointing toward bottom of filter slider.
Bottom of Filter Slider
Filter Wavelength Labels
Color-coded Dots

Figure 67. Emission Filter and Slider

Installing Excitation Filters into an Excitation Filter Slider

Excitation filters have no orientation requirements, but they are usually installed so that their part numbers and wavelength are visible through the hole in the bottom of the slider.

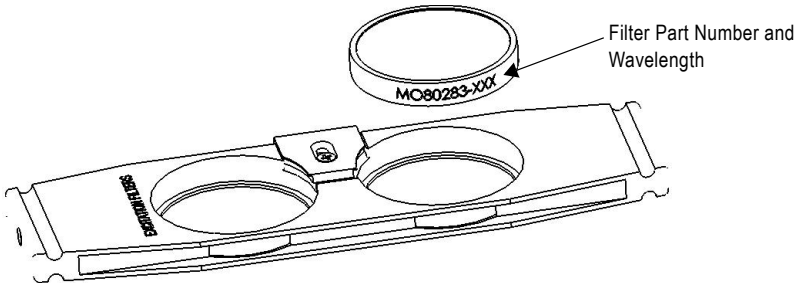


Figure 68. Excitation Filter and Slider

Removing Filters from a Slider

To remove a filter from the slider, loosen the screw holding the filter keeper in place (no need to completely remove the screw) and slide the keeper out of the way. Remove the filter by either pushing it up from below with a tissue, or by carefully turning the slider over and allowing the filter to drop out into your hand. Be careful not to touch the glass with your hand.

Cleaning Filters and Other Optical Surfaces

If you need to clean any optical surfaces, apply lens-cleaning fluid, spectroscopic-grade isopropyl alcohol, distilled water, or methanol to a lint-free lens tissue and drag-wipe the surface.

Do not rub the surface because anti-reflection coatings can be scratched. Repeat with a fresh tissue and fluid after one pass, since contaminants need to be wicked away, not spread over the optical surface.

Appendix D. CRi Software End-User License Agreement

The following is an agreement (the "Agreement") between you and Cambridge Research & Instrumentation Inc., 35-B Cabot Road, Woburn, MA 01801 ("CRi") for software known as Maestro and its accompanying documentation (collectively, the "Software"). By installing and/or using the Software, you agree to the following terms and conditions. If you do not agree to all of the terms and conditions in this Agreement, you may not install or use the Software.

Single Use License. The Software is licensed to you and not sold. Subject to the terms and conditions of this Agreement, CRi hereby grants to you a restricted, nonexclusive, nontransferable, nonassignable, nonsublicensable and revocable license to use, for your internal purposes only, the executable code version of the Software and the accompanying documentation in hard copy or electronic format. CRI RESERVES ALL RIGHTS NOT EXPRESSLY GRANTED BY THIS AGREEMENT.

Specific Restrictions. You may use the Software only on a single computer at a time. You may not use the Software on more than one computer or computer terminal at the same time, nor allow access to the Software from one computer to another over a network, the Internet or by any other means. You may make only one (1) copy of the Software, solely for backup purposes. You may not decompile, reverse engineer, disassemble, alter, modify, translate, adapt, decipher, or determine the source code or create derivative works of, the Software.

Ownership of Software. CRi and/or its suppliers own all right, title and interest, including all copyrights, in and to the Software. The Software contains confidential information and trade secrets of CRi. You (i) acknowledge, and agree not to contest, CRi's rights in the Software; (ii) agree not to disclose to anyone, or allow anyone access to, the Software; and (iii) agree not to disclose any confidential information of CRi regarding the Software or that is otherwise disclosed to you in connection with this Agreement.

Transfer Restrictions. The Software is licensed only to you. The Software may not be transferred to anyone without the prior written consent of CRi. The terms and conditions of this Agreement shall bind any CRi-authorized transferee of the Software. You may not transfer, assign, rent, lease, lend, sell, grant a security interest in, sublicense or otherwise dispose of the Software, on a temporary or permanent basis, except for a CRi-authorized transfer as stated above.

Termination. The license granted in Section 1 above is effective until terminated. This Agreement is conditioned upon your continued compliance with the terms and conditions hereof and will terminate automatically without notice from CRi if you fail to comply with any term or condition of the Agreement. Furthermore, CRi may terminate this Agreement at any time upon thirty (30) days notice. Upon termination of this Agreement, you shall immediately destroy all copies of the Software (including all accompanying documentation) and any other confidential and proprietary information you have received during or in connection with this Agreement.

Limited Warranty. CRi warrants that the media on which the Software is provided will be free from defects in materials and faulty workmanship under normal use for a period of ninety (90) days from the date of delivery. Your exclusive remedy under this Section 6 shall be, at CRi's option, a refund of the price paid for the Software or replacement of the media on which the Software was provided so long as that media has been returned to CRi under a CRi-issued return authorization. CRi shall have no responsibility to replace media damaged by accident, abuse or misapplication.

No Other Warranties. EXCEPT FOR THE LIMITED WARRANTY STATED IMMEDIATELY ABOVE, THE SOFTWARE IS PROVIDED “AS IS” WITHOUT WARRANTY OF ANY KIND, AND CRI EXPRESSLY DISCLAIMS ANY AND ALL IMPLIED WARRANTIES, INCLUDING WITHOUT LIMITATION ANY WARRANTY OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE OR NONINFRINGEMENT OF THIRD PARTY RIGHTS. CRI DOES NOT REPRESENT OR WARRANT THAT THE RESULTS OR THE USE OF THE SOFTWARE WILL BE CORRECT, ACCURATE OR RELIABLE, OR THAT THE SOFTWARE WILL OPERATE UNINTERRUPTED OR ERROR-FREE, OR THAT DEFECTS IN THE SOFTWARE WILL BE CORRECTED. YOU ASSUME ALL RISK ASSOCIATED WITH THE USE, RESULTS AND PERFORMANCE OF THE SOFTWARE.

Limitation of Liability. IN NO EVENT SHALL CRI, ITS AFFILIATES OR SUPPLIERS, OR THEIR RESPECTIVE EMPLOYEES, OFFICERS OR AGENTS, BE LIABLE FOR ANY DAMAGES ARISING OUT OF THE USE OR INABILITY TO USE THE SOFTWARE, INCLUDING WITHOUT LIMITATION INCIDENTAL, SPECIAL, CONSEQUENTIAL, PUNITIVE, EXEMPLARY, INDIRECT OR DIRECT DAMAGES (INCLUDING WITHOUT LIMITATION DAMAGES FOR LOSS OF PROFITS, LOSS OF DATA, RE-RUN TIME, INACCURATE INPUT, WORK DELAYS, BUSINESS INTERRUPTION OR ANY OTHER COMMERCIAL DAMAGES OR LOSSES), WHETHER IN AN ACTION IN CONTRACT, TORT (INCLUDING NEGLIGENCE AND STRICT LIABILITY) OR OTHERWISE, AND EVEN IF CRI HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES, AND REGARDLESS OF WHETHER ANY REMEDY FAILS OF ITS ESSENTIAL PURPOSE.

U.S. Government End Users. The Software qualifies as commercial computer software for purposes of the Federal Acquisition Regulations (FAR) §52.227-19 and the Department of Defense Supplement to the FAR (DFARS) §52.227.7013. If the Software is acquired by a civilian government agency, it is furnished with only the minimum Restricted Rights provided by FAR 52.227-19. If the Software is acquired by a military agency, it is furnished with only the minimum Restricted Rights provided by DFARS 52.227-7013(c)(1)(ii).

Miscellaneous. This Agreement contains the entire agreement of the parties with respect to the subject matter hereof and supersedes any proposal or prior agreement, written or oral, and any other communications between the parties relating to the subject matter hereof. No modification or waiver of any provision of this Agreement shall be effective unless in writing and signed by the parties. No delay or failure on the part of any party in exercising any right under this Agreement shall impair any such right or any remedy of such party, nor shall it be construed to be a waiver of any continuing breach or default under this Agreement. In the event any provision of this Agreement is held to be unenforceable, the remaining provisions of this Agreement will remain in full force and

effect. This Agreement shall be governed by the laws of the State of New York without regard to principles of conflicts of laws. Any disputes relating hereto shall be adjudicated only in the state or federal courts in New York County, New York State, and you hereby consent to the exclusive jurisdiction of those courts. This Agreement shall not be governed by the United Nations Convention on Contracts for the International Sale of Goods, the application of which is expressly excluded. You may not assign or otherwise transfer this Agreement or any of your rights or obligations therein without the prior written consent of CRi. You may not use the Software for any unlawful purpose nor export or re-export the Software except as authorized by law.

Should you have any question concerning this Agreement, you may contact CRi by writing to CRi, 35-B Cabot Road, Woburn, MA 01801. You may also call 1-800-383-7924 in the US or +1-781-935-9099 elsewhere.

Notice to Purchaser

The CRi Maestro *in-vivo* imaging system has many applications involving a variety of materials, such as probes, cell lines, animal specimens, etc. Certain of these applications and/or materials may require licensing under patents held by third parties. For example, while AntiCancer Incorporated and Xenogen Corporation do not have U.S. patent rights covering *in-vivo* imaging of conjugated fluorescent labels, a license from AntiCancer Incorporated, 7917 Ostrow St., San Diego, CA 92111 and/or Xenogen Corporation, 860 Atlantic Avenue, Alameda, California 94501, may be required to practice imaging, within animals, of cells genetically engineered to produce light-emitting compounds. CRi's sale or other transfer of the Maestro *in-vivo* imaging system does not convey any right or license under such third-party patents. It is suggested, therefore, that users of the Maestro *in-vivo* imaging system consult with counsel to determine whether licensing of such third-party patents is required.

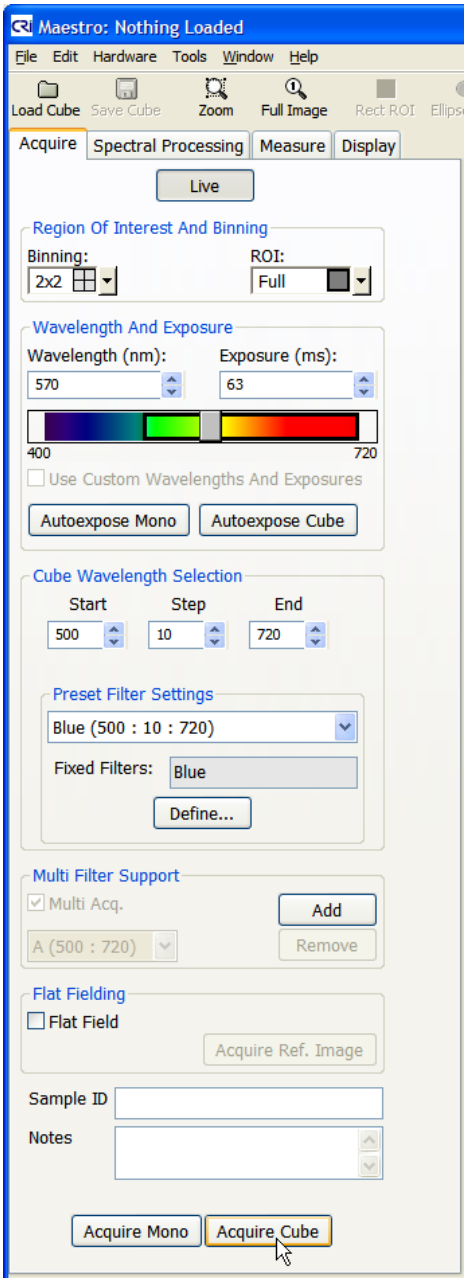
Acquire Images

This guide presumes that the Maestro *In-Vivo* Imaging System is properly installed and configured.

1. Install the excitation and emission filters for the fluorescence you wish to see. (Refer to the Filter Selection Guide in the *"Maestro User's Manual"*.)
2. Switch the main system power **ON** using the switch on the back of the imaging module.
3. With the **SHUTTER** switch in the **CLOSED** position, switch the illumination module **ON** using the switch on the lower right exterior of the imaging module.
4. Switch the interior lights **ON** using the switches on the upper right side of the imaging module.

Remember that if you choose the infrared (**IR**) lights, you will not see any noticeable illumination.

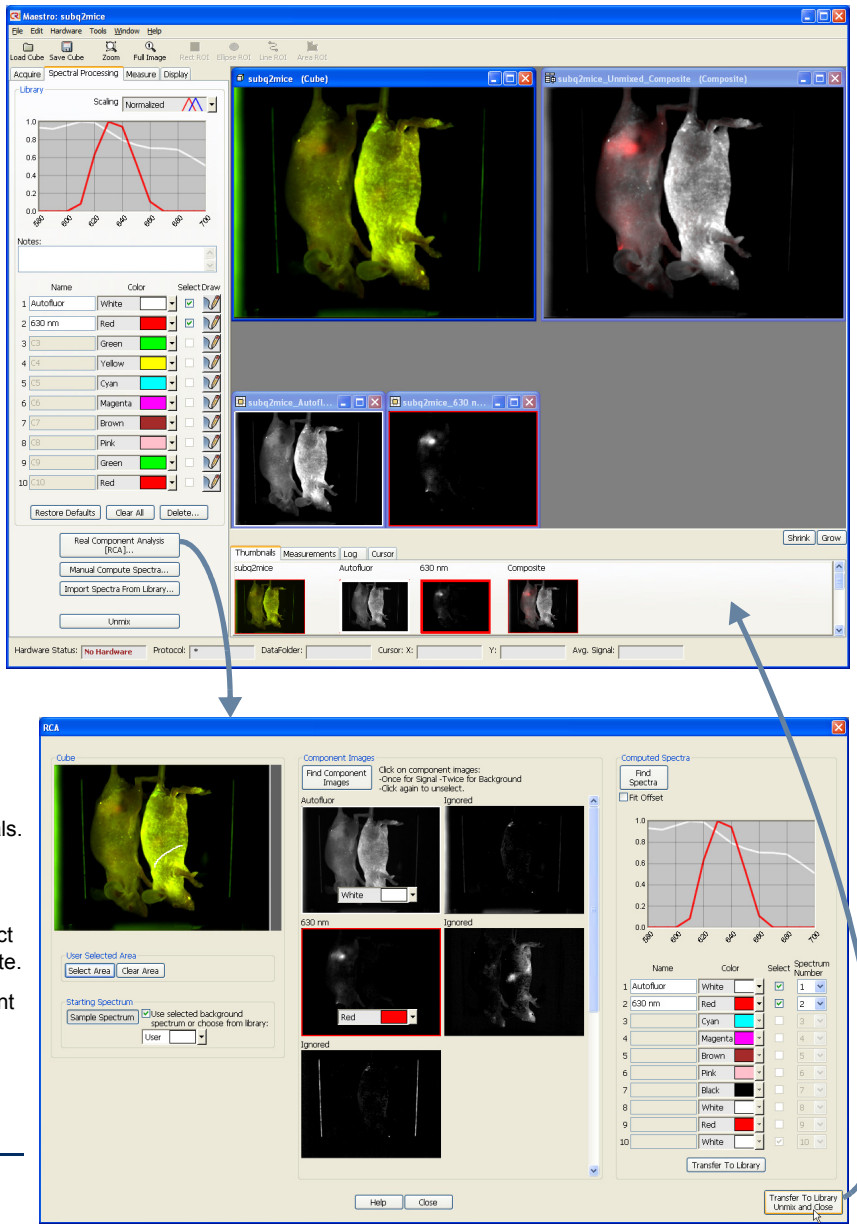
5. Power up the computer and start the **Maestro** application.
6. Place your specimen onto the specimen stage and set the stage and illuminator arms to the heights appropriate for the size of your specimen. Use the field of view markings on the stage surface as a guide to determine the stage and illuminator arm settings.
7. In the Maestro application, select the **Acquire** tab.
8. Click the **Live** button to see live streaming video of the image.
9. Select **Binning** and **Region-Of-Interest (ROI)** options according to the desired image quality. The default is full-field and 2x2 binning. Image files will be smaller with smaller ROI and increased binning. Binning increases sensitivity to light but reduces image resolution.
10. In the **Wavelength and Exposure** box, enter the wavelength at which you expect to see an image of your specimen. This should correspond to the installed emission filter.
11. Click the **Autoexpose Mono** button to improve the live image.
12. Open the door and focus the lens to get a sharp image of your specimen. (If the live image becomes saturated with light when the door is open, it may be difficult to focus the image properly. Try autoexposing the image again with the door open.)
13. Close the imaging module door. Autoexpose the image again if you had to change the exposure setting while focusing.
14. In the **Cube Wavelength Selection** box, select the **Preset Filter Setting** that corresponds to the installed filters. If you don't find a preset that works for your specimen, you can edit the **Start**, **Step**, and **End** settings manually. Refer to the *"Maestro User's Manual"* for instructions.
15. Switch all interior lights **OFF** and **OPEN** the excitation light shutter to illuminate the specimen with the excitation light.
16. Click the **Autoexpose Cube** button to automatically calculate the exposure settings for the image cube. You can also adjust the exposure time manually:
    a. If the image is too dark, increase the exposure time in the **Time (milliseconds)** box.
    b. If the image is too bright or saturated (indicated by solid red pixels), reduce the exposure time.
17. Click the **Acquire Cube** button to generate an image cube.
18. When Maestro has finished generating the image cube, a color representation of the cube displays in the image viewing area.
19. Click the **Save Cube** button on the toolbar to save the cube. Select a location and enter a file name. (File name format suggestion: *project_sample_operator_ datetime*).
20. Select a cube type option:
    a. **Image Cubes** saves the cube in CRi format, which includes hardware and display settings for the cube.
    b. **TIFF Cubes** saves the cube as a series of TIFF images with the assigned file name plus an appended number indicating the wavelength for each image in the cube.
21. Unmix the cube as explained on the reverse side of this Quick Start Guide.



Unmix Images

1. In the Maestro application, select the **Spectral Processing** tab.
2. Open or acquire an image cube. To open a cube, click the **Load Cube** button on the toolbar. Cube format types include CRi format (*.im3) cubes and TIFF (*.tif) cubes. An RGB color representation of the cube will display in the Maestro image viewing area.
3. If a **Live Stream** window is still open, you may close it by clicking its **Close** box.
4. If desired, select a spectral display option from the **Scaling** combo box.
5. Click the **Real Component Analysis (RCA)** button near the bottom of the **Spectral Processing** panel. The RCA function automatically detects and separates each of the component signals in the cube.

If you prefer to unmix the cube by manually identifying the component signals, populate the color library with spectra and click the **Manual Compute Spectra** button. Refer to the *"Maestro User's Manual"* for instructions.



6. In the **RCA** dialog box, you have the option of manually sampling the autofluorescence. Click the **Sample Spectrum** button and use the mouse pointer to draw a sampling line on an area of the specimen that contains only autofluorescence. (Hold down the left mouse button and drag the pointer to draw.)
7. Click the **Find Component Images** button. Maestro identifies and displays the autofluorescence and fluorescence signals.
8. Select the signals you want to unmix:
   a. The autofluorescence is displayed in the upper left image. Click two times anywhere in the image to select it as the autofluorescence. Set its pseudo color to white.
   b. Single-click on the other component image(s) you want to unmix, and select a pseudo color for each.
9. Extract the pure fluorescence signal(s) from the autofluorescence by clicking the **Find Spectra** button. The component signals display in the spectral graph.

Occasionally, for weak signals, there may be too much baseline offset in the data. If you did not get the desired unmixing results, select the **Fit Offset** option and see if the unmixing results improve.

10. Use the controls in the **Computed Spectra** box to specify each spectrum's name, pseudo color, and location within the spectral library.
11. When finished, click the **Transfer to Library, Unmix and Close** button. This adds the spectra to the spectral library and unmixes the spectra in the cube. (If a library is open already, you may be asked if you want to overwrite the existing spectra.)
12. New images will display in the image viewing area:
    a. There is a small component image for each selected spectrum, each with a colored border that corresponds to the pseudo color of the spectrum.
    b. There is also a larger pseudo-colored composite image displayed beside the original RGB image of the cube. The colors used to create this image are library pseudo colors. The result should be a clear display of the specific fluorescence signal, with obvious differentiation from the autofluorescence.
13. Save the resulting images:
    a. You can save all images as displayed by selecting **File > Save Image > Save All (As Displayed)**. Select or create a folder in the same directory as the original image cube. Images are saved as TIFF images that can be opened in a variety of image display programs.
    b. You can save all images as unscaled data by selecting **File > Save Image > Save All Images (As Unscaled Data)**. See the *"Maestro User's Manual"* for more about saving cubes and images.
    c. You can also save the entire workspace by selecting **Save Result Set** from the **File** menu. Enter a file name to save all images and results in a single file.
14. Save the Protocol and/or Spectral Library for use throughout your experiment:
    a. You can save the complete Maestro protocol, which includes the current spectral library, by selecting **Save Protocol** from the **File** menu.
    b. If you want to save the spectral library as a separate file, select **Save Spectral Library** from the **File** menu.

Index

A

absorption spectrum 4
acquire cube 31, 58
acquire mono 58
acquire time sequence 59
acquisition time (typical), specification 91
acquisition wavelength 52
altitude, operating specification 91
autoexpose at current wavelength (mono) 53
autoexpose cube 53
autoexpose target % of dynamic range 27
autofluorescence 5
automatic save options 61

B

bandwidth, specification 91
batch processing 72
binning 51, 81
bit depth 26
brightness level 85

C

calibrating pixel size 104
CCD sensor active area 89
CE test 12
Cleaning
  filters 109
  system 22
clone, region 80
colors, eye's ability to distinguish 3
component selection 88
Compute Spectra
  manual 68
  using RCA 65
contrast level 85
control panels, introduction 23
cooling, specification 91
CSA mark 12
Cube
  acquire 31, 58
  autoexpose 53
  definition of 7
  importing into result sets 71
  information, viewing 61
  save 60
  save notes with 59
  save sample ID with 59
  subtracting spectra 72
  unmix 32
  zooming in/out 25
Cube Wavelength Selection
  manual adjustment 55
  presets, editing 55
  presets, using 55
current wavelength, specifying 52

D

dark current 27
Disclaimers
  design change 12
  reproduction 12
Display 85
  brightness and contrast 85
  component selection 88
  wavelength to RGB map 87
distance calibration 104
distributors, CRi in the EU 12

E

Electrical Supply
  specification 91
electromagnetic spectrum 2
emission filter slider, inserting 18
emission spectra 4, 6
environment, operating specification 91
error scaling 69
excitation filter slider, inserting 21
Exposure Time
  adjusting 53
  custom settings 53
exposure time ratios 57
extract image plane 62

F
f/stop 30
field of view 15
filter selection guide 107
filter serial port 26
Filters
  choosing 5, 54
  cleaning 109
  excitation and emission 5
  installing/removing in sliders 108
  selection guide 107
fit offset 66, 69
flat fielding 58
fluorescence, defined 3
frames to average 27
frequently asked questions 89
Fuses, replacing
  illumination module 20
  imaging module 14

G
gain 26

H
human perception of light 2
Humidity
  operating, specification 91
  storage, specification 91

I
Illumination module
  fuse replacement 20
  lamp replacement 19
  on/off switch 21
  overview 19
illumination, eye's response to 3
illuminator arms, positioning 16
image cube, defined 7
image data pages, introduction 23
image plane, extract 62
image sensor format, specification 91
image sensor pixel-count, specification 91
Imaging Module
  dimensions, specification 92
  overview of 13
imaging theory 1
Installation
  Maestro software 101
  Maestro system 97

L
Lamp Cartridge
  lifetime, specification 19, 92
Lamp Cartridge, replacing 19
light delivery system, specification 92
light, discussion of 1
lights, interior on/off switch 17
Line Profiles 74
  clone 76
  copy 76
  delete 76
  move 76
live image 51
luminescence, defined 3

M
Maestro *in-vivo* imaging system, introduction to 9
Maestro Work Area
  how to launch 22
  understanding 23
maximum exposure 27
Measurements 77
  appending to a text file 84
  copy to clipboard 84
  definitions 83
  measuring regions 36, 77
  save as text 84
Mechanical Dimensions
  illumination module 94
  imaging module 93
  specimen stage fields of view 95
multi filter protocols 57
multi filter support 55
multispectral analysis, introduction to 7

N
notes, save with cube 59

P
Panning Images
  individual 25
  together 26

phosphorescence, defined 3
pixel size, calibration 104
Power Supply
  on/off switch 14
Protocols 43
  open 50
  save 43
pure spectra, calculating 34

R
ratio of exposure times 57
real component analysis (RCA) 65
Region
  clear all 79
region of interest (ROI) 52
region of interest shapes 68
Regions
  clone 80
  copy all 80
  copy measurements 84
  delete 79
  drag a copy 80
  erase part of 79
  find automatically 77
  ignoring small 82
  labels 82
  load to an image 81
  manual draw 79
  measurements 83
  measuring 77
  modify 79
  move 79
  save 81
  saving measurements 84
  threshold segmentation 77
  transparency and color 82
reinitialize hardware 28
resolution, low 27
result set, saving 70

S
Safety
  cautionary statements 10
  operator and equipment 10
  technical assistance contact 11
sample area, specification 91
sample ID, save with cube 59
saturation mask 86
Save
  cube 60
  image 61
  region 81
  unmix results 70
snap shot 58
software license agreement 111
Specifications
  mechanical dimensions 93
  operating 91
Specimen Stage
  height setting 81
  positioning 15
Spectra
  computing manually 68
  import from component image 44
  import from composite image 45
  import from library 44
  sampling manually 67
  subtracting from cube 72
Spectral Graph Scale
  normalized 70
  scale to max 70
  un-normalized 70
Spectral Library
  open 64
  save 43
Stoke's Shift 4

T
Temperature
  operating, specification 91
  storage, specification 91
third-party computers, using 105
threshold segmentation 77
thumbnails, introduction to 23
timed acquisition 59
timed sequences 59

U
UL test 12
unmix cube 32
user's manual, about 11

V
visible wavelength, discussion of 2

W
wavelength and exposure 52
wavelength range, specification 91
wavelength slider, using 52
wavelength to RGB map 87
weight, system specification 92
window layout, description 24
windows, resizing 25
Workstation Computer
  overview of 22
  specifications 92

Z
zooming images 25

Warranty

CRi warrants its Maestro systems for a period of one (1) year from date of shipment against defects in material and/or workmanship, provided its installation, application, and maintenance are within specifications. Normal wearing parts are excluded. This warranty covers only items manufactured by CRi. CRi will correct, by repair or replacement-at its option and its expense-any proved defects in items of its manufacture, subject to above, provided immediate written notice of such defects is given.

A valid Return Materials Authorization (RMA) must be obtained by contacting CRi before items may be returned to the factory. Repair or replacement will be provided F.O.B. (Freight On Board) at CRi's factory. The total financial obligation of CRi, under this warranty, does not exceed the purchase price of the items of its manufacture as set forth on normal pricing schedules. We will not assume any expense or liability for repairs made by others without our prior written consent.

This warranty is void and CRi will be free from all obligations hereunder if the items of its manufacture have been misused, reprocessed or reused, repaired or modified without our written consent. CRi assumes no liability for damages or injuries resulting from the misuse, misapplication, or unauthorized repair or modification of its equipment.

We assume no liability to users of our equipment or to any other person or persons for special or consequential damages of any kind and from any cause arising out of or in any way connected with the use of the equipment. All warranty and post-warranty service is nontransferable from the original shipping address to another without the prior written consent of CRi.

The CRi Maestro system may be used for research purposes. It is not intended for clinical or diagnostic use at this time.

TO PLACE AN ORDER OR RECEIVE TECHNICAL ASSISTANCE

For more information, contact CRi or your local authorized CRi distributor:

Cambridge Research & Instrumentation, Inc. (CRi)

35-B Cabot Road, Woburn, MA, 01801, USA

(Toll-Free US) 1-800-383-7924, (Phone) +1-781-935-9099

(Fax) +1-781-935-3388 (Email) techsupport@cri-inc.com or sales@cri-inc.com

Maestro and Nuance are trademarks of Cambridge Research & Instrumentation, Incorporated.

Adobe and PhotoShop are trademarks of Adobe Systems, Incorporated.

Alexa Fluor and MitoTracker are trademarks of Molecular Probes, Inc.

AntiCancer is a trademark of AntiCancer, Incorporated.

Cermax is a trademark of PerkinElmer, Incorporated.

Cooper Bussman is a trademark of Cooper Industries, Inc.

Cyanine dye (Cy2, Cy3, Cy5, Cy5.5 and Cy7) is a trademark of Amersham Biosciences.

Intel, Centrino, and Pentium are trademarks of Intel Corporation or its subsidiaries in the United States and other countries.

Invitrogen is a trademark of Invitrogen Corporation.

Microsoft, Windows, and Excel are trademarks of Microsoft Corporation in the United States and other countries.

QDot is a trademark of Invitrogen Corporation.

Xenogen is a trademark of Xenogen Corporation.

© 2006 Cambridge Research & Instrumentation, Inc. All rights reserved.

Document Part No. MD15379 Rev. B, August 2006